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Metal complexes of O-phosphorylserine

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With 4 figures in the text

The wide distribution of O-phosphorylserine as metabolic key-compound (1–4) and its multidentate character, including the phosphate, carboxyl and amino donor groups will lend itself very suitably as a model for many important biochemical compounds in the study of their metal complexity. Such a study is of interest, as certain enzymatic (5–7) and non-enzymatic (8) reactions, involving phosphoryl groups, are activated by metal ions. The action of the metal ion in these reactions is considered to take place partly through complex formation with the substrate (6–8). Some phosphoseryl compounds, phosphopeptides derived from cow's casein, may also, as pointed out by Mellander (9), be of great physiological interest due to complexing ability, and *in vivo* act as vehicles for metals of physiological importance. In the present study apparent stability constants of some metal complexes of phosphorylserine and some ionization constants of this compound will be reported.

The acid ionization and the complex formation were measured by pH titration according to Bjerrum (10, 5 pp. 78–94). The stability constants were computed from values on the hydrogen concentration, the concentration of free ligand, A, and the degree of complex formation, Z. The values of A and Z were obtained from the titration data, using ionisation constants previously computed.

A preliminary account of the present investigation has been given earlier (11).

Experimental

Materials

O-phosphorylserine was synthetized by an adaption of the method described by Canali *et al.* (12) as follows: 40 cc 85 % H_3PO_4 (Merck p.a.) was heated to 350°C and then left to cool to 100°, after which racemic serine was added. The resulting syrup was then treated for 30 min. at 125–130°, cooled to about 60° and precipitated with cold acetone (5°). After reprecipitating with acetone, the product was dissolved in 200 cc. 2 M HCl and was refluxed for 2 hours. The hydrochloric acid-treated solution was then mixed with bariumhydroxide to an alcaline reaction and subsequently brought to pH 9 by ammonia. This resulted in precipitation of mainly inorganic phosphates, which were separated by centrifugation. After washing the precipitate once, the combined supernatants were adjusted to neutral pH. After evaporating the resulting solution to a small volume, it was precipitated by absolute ethanol (v/v = 1/1). The precipitate was washed three times with 50 % ethanol, and then dissolved to a small volume by aq. dest. This solution was then subjected to ion

exchange chromatography (Dowex 50-X8 in H^+-form) on a 44 × 2 cm column, being eluated with 0.01 M HCl. After 120 ml of eluate, the only ninhydrinpositive fraction came out, and this was evaporated to one third of the volume. On adding ethanol to the evaporated solution, PS[1] was crystallized and then recrystallized from an ethanol-water mixture.[2] Yield: 0.85 g. Analyses: calculated for $C_3H_8O_6NP$; C, 19.4; H, 4.3; N, 7.57; P, 16.8; found:[3] C, 19.4; H, 4.4; N, 7.4; P, 17.2.—The purity[4] was further checked by: 1) paper chromatography in the following solvents: phenol/water (v/v = 80/20), *t*-butanol/6 M HCl/water (v/v/v = 21/4/5) and *t*-butanol/propionic acid/water (v/v/v = 40/30/30). On each occasion PS furnished a single spot on the chromatogram, indicated by ninhydrin, and the phosphate spot test of Hanes and Ischerwood (15). 2) Potentiometric titration. Calculated mol.wt. 185.1, found 185.2.

Aqueous solutions of PS, 1.355×10^{-2} M and 4.12×10^{-3} M, were prepared and potentiometrically standardized. Metal solutions of the molar concentrations 1.4×10^{-2} and 8×10^{-2} were made from $CaCl_2 \cdot 6H_2O$, $MgCl_2 \cdot 6H_2O$, $MnCl_2 \cdot 4H_2O$ and $CuCl_2 \cdot 2H_2O$ (an 1.4×10^{-2} M solution only). For the Fe(III) titrations 2×10^{-3} and 1.2×10^{-3} M solutions were prepared from $FeCl_3 \cdot 6H_2O$. The metal chloride solutions were standardized by chloride titration according to Mohr (16). The Ca(II) solutions were further standardized by complexometric titration with ethylendiaminetetraacetic acid (17). The Fe(III) and Mn(II) solutions were never used later than 4 hours after the preparation. All metal solutions were brought to a final ionic strength of 0.30 by adding KCl-solutions (v/v = 1/1) of suitable concentrations. Carbonate-free 0.1 M KOH was prepared according to Schwarzenbach *et al.* (18) and was standardized against potassium hydrogen phthalate. 0.1 M HCl was prepared from constant boiling hydrochloric acid and standardized against potassium iodate. All chemicals were of analytical grade and were used without further purification. Redistilled water was used throughout.

Apparatus

The titrations were carried out in a 70 ml multineck beaker designed to accomodate electrode, microburette, thermometer, and gas-inlet. The titration vessel was kept in an oil thermostate at 25.0 ± 0.2°C, and the atmosphere was kept constant by bubbling nitrogen, saturated with water vapour by bubbling through 0.15 M KCl in the thermostate and rinsed from droplets by a cotton wool filter. Measurements of the hydrogen concentration were performed by a Radiometer glass electrode of shielded type (G 102 B) and a calomel electrode (K 100), recorded by a valve potentiometer (type Radiometer PHM 3). The electrode assembly was standardized with 0.05 M potassium hydrogen phthalate and 0.05 M borax according to Hitchcock *et al.* (19). The pH measurements could in general be reproduced within 0.01 in pH.

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[1] PS = O-phosphorylserine.

[2] As the recrystallization was rather rapid and completed with an excess of ethanol, no crystal water could be detected. Slow crystallization will produce one crystal water per molecule PS (13).

[3] The analyses of C and H were performed by Mikroanalyslaboratoriet, Medicinsk-Kemiska Institutionen, Uppsala and the N- and P analyses were performed by a standard Micro-Kjeldahl method and a method described by Svennerholm, respectively (14).

[4] The infrared spectroscopy curve and the X-ray diffraction pattern of PS, prepared in the way described but crystallized with one mole of crystal water per mol PS, were the same as PS prepared by an other route (13).

Procedure

A 10.00 ml aliquot of PS stock solution was transferred to the titration vessel followed by 10.00 ml metal solution. For the investigation of hydrogen equilibria, 0.30 M KCl solution was added instead of the metal solution. For the 1:4 (metal: ligand) titration of the ligand in the presence of Fe(III), 3.00 ml of the strongest concentrated ligand solution was mixed with 10.00 ml iron solution and then made up to 20.00 ml by aq. redest. When thermal equilibrium was attained, 0.1 M KOH or 0.1 M HCl was delivered, the pH of the solution being recorded for each addition. Stirring was achieved by the nitrogen bubbling. The procedure was continued to pH 10.5 or to pH 2. Every titration type was reproduced at least once. In this way 1:2 and 9:1 (metal:ligand) titrations were performed with Ca(II), Mg(II) and Mn(II), 1:2 titrations with Cu(II) and 1:4 and 1:7 titrations with Fe(III).

To convert the pH-meter readings to hydrogen concentration, the activity coefficient of Harned and Hamer (20) as stated for 0.15 M KCl were used in the pH range 3–9 ($\gamma = 0.77$). Outside this pH-region, the readings were calibrated directly from a hydrochloric acid-potassium hydroxide titration curve, obtained by the titration of an aqueous blank according to the above-mentioned conditions. As the ionic strength and the volume of the added reagent were kept comparatively small, this calibration seems valid (21). The value of the ionzation product of water ($=1.008 \times 10^{-14}$) and the value of the ionic activity coefficient product of water ($=0.59$), which were used, were taken from Harned and Hamer (20). It was assumed that any influence of the complex forming ions upon the medium could be neglected, and that the bulk electrolyte did not participate in the complex formation in any measurable quantities.

Calculation of the constants

List of symbols

A	Ligand, completly ionized.
A	Molar concentration of free ligand.
a	Number of proton sites of the ligand not being occupied at electric neutrality.
b_n	Apparent stability constant of the complex MA_n, defined by $b_n = [MA_n][MA_{n-1}]^{-1}\ M^{-1}$.
b_{MHA}	Apparent stability constant of the complex MHA, defined by $b_{MHA} = [MHA][HA]^{-1}\ M^{-1}$.
b_{M_2A}	Apparent stability constant of the complex M_2A, defined by $b_{M_2A} = [M_2A][MA]^{-1}\ M^{-1}$.
b_{MOH}	Equilibrium constant defined by $b_{MOH} = h[MOH]\ M^{-1}$.
C_A	Total molar concentration of the ligand.
C_M	Total molar concentration of the metal ions.
j	Number of protons bound in the acid H_jA.
J	Maximum value of j.
C_S	Difference between the molar concentration of bound protons and bound hydroxyl ions, defined by $C_S = C_A(J-a) - [KOH] + [HCl] - h + [OH]$.
h	Molar concentration of free hydrogen ions.
K_{H_jA}	Apparent ionization constant of the acid H_jA, defined by $K_{H_jA} = h[H_{j-1}A][H_jA]^{-1}$.
K_{MHA}	Apparent ionization constant of the acid MHA defined by $K_{MHA} = h[MA][MHA]^{-1}$.
M	Complex forming central group.
M	Molar concentration of free metal ions.
n	Number of A groups bound in the complex MA_n.
N	Maximum value of n.

$\bar{n}_H$ Average number of protons bound per C_A, defined by $\bar{n}_H = C_S\ C_A^{-1}$ for solutions, where no complex forming metal ions are present.

P $= b_1 b_{MHA}^{-1}$.

pK_{H_jA} $= -\log K_{H_jA}$.

t Number of A groups bound in the complex MA_t.

Z Average number of ligands bound per C_M, defined by eq. 6.

β_n $= \prod_0^n b_n$; $\beta_0 = 1$.

$\varkappa_j$ $= \prod_0^j K_{H_jA}$; $\varkappa_0 = 1$.

[ ] Molar concentration of the species, indicated.

$Y(X)_Q$ Indicates that Y is a function of X, where Q is considered a constant.

The charges of the ions have been omitted for the sake of convenience.

Acid ionization constants

The ionization of a j-basic acid may be formulated

$$H_jA \rightleftharpoons H_{j-1}A + H; \quad 1 \leqslant j \leqslant J$$

As no complex forming metal ions are present,

$$C_S = \sum_{j=1}^{J} j\,[H_jA]. \tag{1}$$

If the ionization steps are sufficiently separated, any pK-value of the polybasic acid H_jA may be calculated within ± 0.01 in pK by the equation (cf. 10, p. 205)

$$pK_{H_jA} = -\log h + \log \frac{\bar{n}_H - j + 1}{j - \bar{n}_H}. \tag{2}$$

The figure $\bar{n}_H - j + 1$ of equation 2 had to be in the range 0.10–0.90, and $-\log h$ may be $> pK_{H_{j+1}A} + 2.7$ and $< pK_{H_{j-1}A} - 2.7$.

Stability constants

C_M and C_A were known at all titrations and they were only slightly changed during each titration. C_S and h were varied and determined from the experimental measurements.

For decreasing values of h, a step-wise complex formation in the order MHA, MA, MA_2 ... MA_N may be valid. If MOH and M_2A species are also considered as possibly existing, C_M, C_A, C_S and Z are given by (cf. 10, 22):

$$C_M = M\,(b_{MHA} K_{HA}^{-1} h\, A + \sum_{n=0}^{N} \beta_n A^n + b_{MOH} h^{-1} + 2\, b_1\, b_{M_2A}\, A\, M) \tag{3}$$

$$C_A = M\,(b_{MHA} K_{HA}^{-1} h\, A + \sum_{n=1}^{N} n\beta_n A^n + b_1\, b_{M_2A}\, A\, M) + \sum_{j=0}^{J} [H_jA] \tag{4}$$

$$C_S = M\,(b_{MHA}\,K_{HA}{}^{-1}\,h\,A - b_{MOH}\,h^{-1}) + A\sum_{j=1}^{J} \varkappa_j{}^{-1}\,j\,h^j \tag{5}$$

$$Z = (C_A - \sum_{j=0}^{J} [\mathrm{H}_i\mathrm{A}])\,C_M{}^{-1} \tag{6}$$

$$Z = \frac{b_{MHA}\,K_{HA}{}^{-1}\,h\,A + \sum_{n=1}^{N} n\,\beta_n\,A^n + b_1\,b_{M_2A}\,A\,M}{b_{MHA}\,K_{HA}{}^{-1}\,h\,A + \sum_{n=0}^{N} \beta_n\,A^n + b_{MOH}\,h^{-1} + 2\,b_1\,b_{M_2A}\,A\,M} \tag{7}$$

Eq. 7 may be multiplied out and the terms grouped to give (cf. 10, 23):

$$Z + b_{MOH}\,Z\,h^{-1} = b_{MHA}\,K_{HA}{}^{-1}\,(1-Z)\,h\,A + \sum_{n=1}^{n=t-1} \beta_n\,(n-Z)\,A^n + \beta_t\,(t-Z)\,A^t +$$

$$+ \sum_{n=t+1}^{N} \beta_n\,(n-Z)\,A^n + b_1\,b_{M_2A}\,(1-2\,Z)\,A\,M;\ 1 \leqslant t \leqslant N \tag{7a}$$

Eq. 7a is the equation used to calculate the stability constants.

At the 1:2 (C_M:C_A) titrations of this study, M_2A species may be neglected. The value of b_{MOH} may be taken from the literature, and the figure related to MOH (if considered), therefore will not complicate the calculations. Further, the unknown variables A and Z may be calculated from experimental data and known constants, when a limited number of complexes exists (see below). When h is gradually reduced, the stability constant for each complex may, thus, successively be calculated in a range, where the next complex formed, needs no consideration. At the calculation of any constant b_t from eq. 7a e.g., the terms on the right hand side of the β_t-term will be omitted, and the terms on the left hand side of β_t will be known.

When the MHA-species only are considered, A is obtained by combining eqs. 4 and 5 to (figures related to MOH omitted).

$$A = \frac{C_S - C_A}{\sum_{j=0}^{J} \varkappa_j{}^{-1}\,(j-1)\,h^j} \tag{8}$$

Z may then be calculated from eq. 6.

If the MOH and MA_n species are also considered, A and Z may be solved graphically from eqs. 6 and 9, the latter being derived from eqs. 3–6.

$$Z = t - \frac{\left[C_S - A\sum_{j=0}^{J} \varkappa_j{}^{-1}\,j\,h^j\right]\left[b_{MHA}\,K_{HA}{}^{-1}\,(t-1)\,h\,A + \sum_{n=0}^{n=t-1} \beta_n\,(t-n)\,A^n + t\,b_{MOH}\,h^{-1}\right]}{C_M\,(b_{MHA}\,K_{HA}{}^{-1}\,h\,A - b_{MOH}\,h^{-1})} \tag{9}$$

In the plot $Z(A)_{c_M, c_A, c_S, h}$ for every experimental measurement, the intersection of eqs. 6 and 9 will give actual Z, A values (Fig. 1) for the calculation of any constant b_t. Eq. 6 gives a straight line in this plot and may be conveniently constructed from

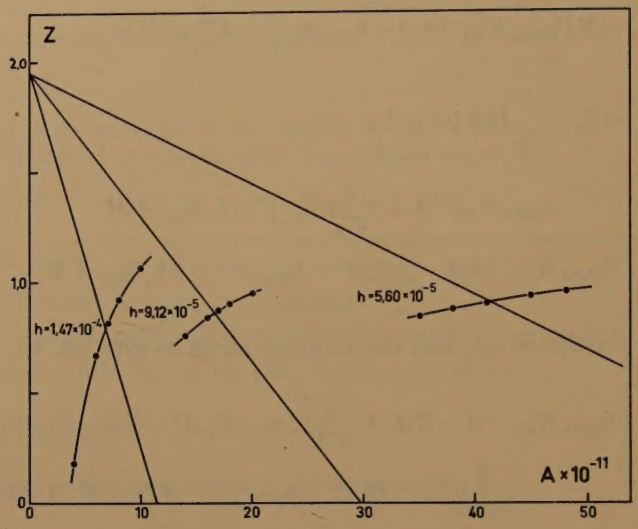


Fig. 1. Finding Z, A from the intersection of eqs. 6 and 9 (figures related to MOH omitted) in the plot $Z\ (A)_{C_M, C_A, C_S, h}$ for the determination of b_1. Values taken from the copper(II) titration.

the intercepts $C_A\ C_M^{-1}$ and $C_A\left(\sum_{j=0}^{J} \varkappa_j^{-1} h^j\right)^{-1}$. By inserting tentative values of A into eq. 9 and solving the corresponding Z values, a sufficient number of A, Z values may be obtained to construct this equation, where it intersects eq. 6. Correction of the eqs. 7a and 9 for the next complex formed may be carried out, when a definite trend appears in the values on the eq. 7a expression, which is related to the constant being computed (Table 1).

At the 9:1 (C_M:C_A) titrations in this study, only formations of the species MHA, MA, M_2A and MOH may be valid. The constant b_{MHA} may still be computed as outlined above. This is true also for b_1, when M_2A species can be neglected. However, A and Z may now more easily be obtained from eqs. 5 and 6, respectively, since M may be regarded as equal to C_M. If M_2A species are formed, the complex MA can probably not be considered separately from M_2A. The computation of b_{M_2A} will therefore need b_1 estimated from the 1:2 titration. Eq. 7a may be used, and values of A and Z may be furnished from eqs. 5 and 6.

At each $-\log h$ value used at the calculations only those H_jA species will be considered, for which $pK_{H_jA} + 2 > -\log h > pK_{H_{j+1}A} - 2$.

Results

Sample potentiometric measurements of corrected pH-values against moles of potassium hydroxide added per mole of ligand present, are given in Figs. 2 and 3. The data thus obtained were used in calculation apparent ioization constants and apparent stability constants. In these calculations the MOH complex was omitted, unless otherwise stated.

Table 1. Computation of the apparent stability constants (in M^{-1}) of the copper (II) complexes at 25°C and 0.15 ionic strength. The numbers in parentheses in the table heads refer to equation numbers.

$C_M = 6.78 \times 10^{-3}$, $C_A = 3.47 \times 10^{-3}$ (initial concentrations).

ml KOH [0.1006 M]	$h \times 10^{-4}$	$A \times 10^{-12}$ (8)	Z (6)	$\frac{Z K_{HA}}{(1-Z) h A} \times 10^4$ (7a)
0.800	21.88	0.393	0.228	6.30
1.000	16.79	0.671	0.286	6.49
1.200	12.05	1.300	0.352	6.36
1.400	7.943	2.92	0.448	6.42
1.600	4.602	8.20	0.570	6.45
1.900	1.469	64.8	0.856	11.42
2.000	0.9120	150.3	0.964	35.8

$b_{MHA} = (6.40 \pm 0.2) \times 10^4$

ml KOH	$h \times 10^{-5}$	$A \times 10^{-9}$ (6, 9)	Z (6, 9)	y_1 [1] $\times 10^9$ (7a)
1.900	14.69	0.0685	0.790	4.40
2.000	9.120	0.166	0.858	4.58
2.100	5.598	0.414	0.908	4.30
2.200	3.475	1.013	0.943	4.18
2.420	1.445	5.20	0.980	4.37
2.600	0.7852	15.40	0.991	4.40
2.800	0.4852	42.9	0.997	6.25
3.000	0.2382	108.4	0.999	8.36

$b_1 = (4.39 \pm 0.2) \times 10^9$

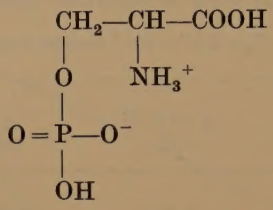
ml KOH	$h \times 10^{-7}$	$A \times 10^{-7}$ (6, 9)	Z (6, 9)	y_2 [2] $\times 10^5$ (7a)
2.800	42.85	0.419	1.018	7.23
3.000	23.82	1.012	1.059	7.62
3.100	17.86	1.48	1.087	7.60
3.200	13.15	2.18	1.125	7.30
3.400	6.903	4.33	1.232	7.40
3.600	3.244	8.04	1.402	8.61
3.700	2.056	10.7	1.510	9.87
3.800	1.189	14.0	1.630	12.3

$b_2 = (7.43 \pm 0.2) \times 10^5$

[1] $y_1 = \frac{Z}{(1-Z)A} - b_{MHA} \frac{h}{K_{HA}}$ [2] $y_2 = \frac{(1-Z)}{(2-Z)A\, b_1} (y_1 - b_1)$

Apparent acid ionization constants

The compound PS



may bind four protons. At electric neutrality, however, PS will probably be a dipole, having taken up three protons as indicated above. The results of two titrations with different ligand concentration are listed in Table 2. It is evident from this table that the uptake of the fourth proton by the ligand was not detected above pH 2. The first ionization of the ligand was therefore not thought to interact at pH ranges, where the complex formations were measured (see below).

Table 2. Computation of apparent pK-values for phosphorylserine at 25°C and 0.15 ionic strength.

$C_A = 6.78 \times 10^{-3}$ (initial concentration)

ml KOH (0.1006 M)	$h \times 10^{-3}$	$\bar{n}_H$	pK_{H_3A}
0.000	4.188	2.380	2.16
0.503	2.541	2.244	2.10
0.703	1.936	2.185	2.07
0.800	1.656	2.152	2.03
0.900	1.337	2.125	2.03
			Av. 2.08 ± 0.08

ml KOH	$h \times 10^{-6}$	$\bar{n}_H$	pK_{H_2A}
1.800	4.539	1.665	5.65
2.000	2.477	1.515	5.63
2.100	1.828	1.440	5.63
2.200	1.337	1.368	5.64
2.475	0.4276	1.162	5.65
			Av. 5.64 ± 0.01

ml KOH	$h \times 10^{-10}$	$\bar{n}_H$	pK_{HA}
3.205	3.055	0.636	9.76
3.400	1.738	0.494	9.75
3.500	1.349	0.429	9.75
3.600	1.040	0.363	9.74
3.800	0.5970	0.242	9.73
			Av. 9.74 ± 0.02

$C_A = 2.06 \times 10^{-3}$ (initial concentration)

ml HCl (0.1010 M)	$h \times 10^{-3}$	$\bar{n}_H$	pK_{H_3A}
0.000	1.702	2.171	2.08
0.200	2.576	2.230	2.06
0.400	3.435	2.285	2.06
0.599	4.285	2.330	2.06
0.800	5.140	2.370	2.06
1.001	5.957	2.420	2.09
1.200	6.777	2.453	2.09
			Av. 2.07

ml KOH (0.1028 M)	$h \times 10^{-6}$	$\bar{n}_H$	pK_{H_2A}
0.500	6.353	1.751	5.68
0.599	2.203	1.505	5.67
0.699	0.7378	1.258	5.67
			Av. 5.67 ± 0.01

ml KOH	$h \times 10^{-10}$	$\bar{n}_H$	pK_{HA}
0.899	6.473	0.775	9.73
0.950	3.811	0.658	9.70
0.999	2.472	0.550	9.69
1.054	1.633	0.437	9.67
1.102	1.178	0.345	9.65
			Av. 9.69 ± 0.04

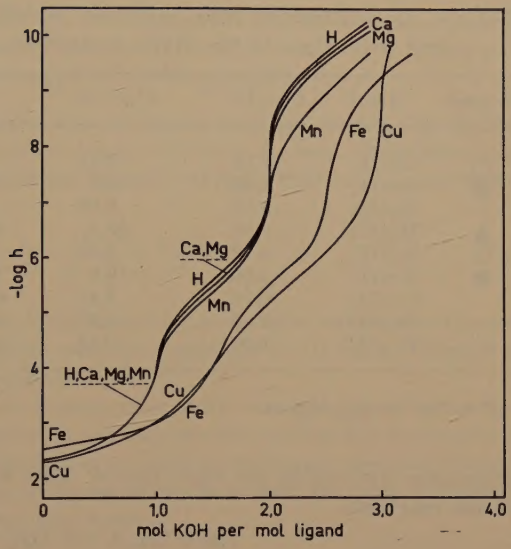


Fig. 2. Titration curves for the ligand and for the 1:2 (C_M:C_A) titrations of Ca(II), Mg(II), Mn(II) and Cu(II), using the initial value of $C_A = 6.78 \times 10^{-3}$. Titration curve for the 1:4 titration of Fe(III), using the initial value of $C_A = 2.03 \times 10^{-3}$.

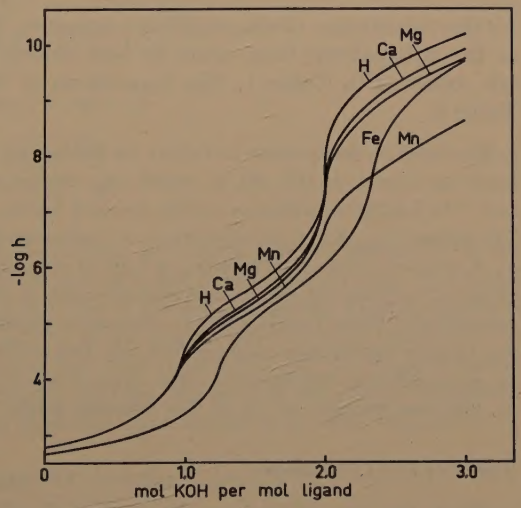


Fig. 3. Titration curves for the ligand, for the 9:1 (C_M:C_A) titrations of Ca(II), Mg(II), and Mn(II) and for the 1:7 titration of Fe(III), using the initial value of $C_A = 2.06 \times 10^{-3}$.

The uncertainty in pK_{H_3A} at the lower value of C_A is reasonably greater than indicated by the individual calculations due to less accuracy in C_S at low pH (cf. 24); e.g. if an error of ± 0.005 in pH is considered, the variation in pK will be within ± 0.08 at the delivery of 1.200 ml HCl.

At the calculation of the stability constants, below, the values of the ionization constants related to the ligand concentration used were chosen.

The Cu(II) complexes

The titration curve, obtained for the 1:2 molar ratio of ligand to copper (II) ions (Fig. 2), rises sharply at about $-\log h = 8$, coinciding with the uptake of 3 moles of

Table 3. Logarithms of the apparent stability constants of the metal complexes and pK-values of the MHA-complexes at 25°C and 0.15 ionic strength.

Symbol	Metal	$C_A \times 10^{-3}$*	$C_M \times 10^{-3}$*	log b_{MHA}	log b_1	log b_2	pK_{MHA}
□	Ca (II)	6.78	3.22	—	2.14±0.10	—	—
■	Ca (II)	2.06	18.8	1.43±0.05	2.19±0.08	—	8.93
△	Mg(II)	6.78	4.00	—	2.39±0.08	—	—
▲	Mg(II)	2.06	19.2	1.60±0.07	2.48±0.04	—	8.81
○	Mn(II)	6.78	3.50	—	3.82±0.09	—	—
●	Mn(II)	2.06	19.9	1.91±0.05	3.92±0.04	—	7.68
	Cu (II)	6.78	3.47	4.81±0.02	9.64±0.02	5.88±0.02	4.91
	Fe (III)	2.06	0.30	6.85	14.15	7.2	2.4
	Fe (III)	2.03	0.48	6.50	13.80	7.6	2.4

* initial concentrations.

potassium hydroxide per mole ligand. This will probably indicate the completeness of the reaction:

$$M + 2H_3A + 6\ OH \rightleftharpoons MA_2 + 3H_2O$$

At the calculation of the stability constants, the MHA-complex was also considered, as the calculations then could be best suited for the experimental data. The results are collected in Table 1. The logarithms of the stability constants are compiled in Table 3.

The trend in the y_2 values in Table 1 for decreasing values of h may be due to formation of Cu(II) hydroxy complexes (25, 26), of which only the mononuclear species, MOH, may be considered (25). The negligible influence of this complex on the calculated data will, however, be apparent. The figures $b_{MHA}K_{HA}^{-1}\,hA$ and $2b_{MOH}\,h^{-1}$, situated within the same brackets in the numerator of eq. 9, are compared at the delivery of 3.80 ml potassium hydroxide. The value of $10^{-8.25}$ for b_{MOH}, obtained by Pedersen (25) at 0.15 ionic strength, is used. The figures will be 58.1 and 9.48×10^{-2}, respectively. Other Cu(II) hydroxy complexes should have influenced the values of y_2 even less, due to their equilibrium constants (25, 26). The trend of the y_2 values may therefore be ascribed to an accumulating experimental error, depending on change of ionic strength, error in b_{MHA} and b_1, and less accuracy of C_S in this titration range (cf. 24).

The Fe(III) complexes

With the use of the same values of C_M and C_A as for the copper(II) titration, a greenish precipitate developed at low pH. This begun to dissolve successively at about pH 5. It was, however, possible to perform Fe(III) titrations up to pH 10 before any visible precipitate developed. In these titrations the values of C_M and C_A, mentioned in the experimental section, were used. The resulting titration curves (Figs. 2 and 3) indicate formation of MA_2* species probably in the same way as the copper(II) curve. This is apparent from the 1:4 and 1:7 (C_M:C_A) titration curves of Figs. 2 and 3 at a value of $-\log h$ of about 7.1 for both, and at the expected

* Formation of the species $(OH)_2$ M $(HA)_2$ would, however, give the same titration curves, but any computation of stability constants have not been carried out for these species in this study.

abscissa values of 2.5 and 2.3, respectively. The ability of PS to bleach a Fe(III) chloride solution and to displace the precipitation of ferric hydroxide to a higher pH constituted further evidence of the formation of stable complexes.

The metal-ligand curve does not confluence with the ligand curve at low pH (Fig. 3). The calculation of the stability constants was therefore begun with the b_2 constant. Considering only the complexes MA and MA_2 as existing, and dividing through with $b_1(2-Z)A^2$, eq. 7a was reduced to:

$$\frac{Z}{b_1(2-Z)A^2} = \frac{1-Z}{(2-Z)A} + b_2 \tag{7b}$$

As the ratio b_1/b_2 was found to be extensive, the b_1 term in this equation was neglected in the range used for the calculation of b_2. This constant was thus equal to $(Z-1)/(2-Z)A$. Values of A and Z were obtained from eqs. 5 and 6 respectively.

The calculations were then continued by the determination of the ratio $b_1/b_{MHA} = P$ in a range, where Z was assumed to be equal to 1, and where it was only necessary to consider the complexes MHA and MA. Formation of MHA species seemed to be valid, as the ligand apparently formed these species with all other metals investigated. By neglecting M as well, the value of P could, thus, be calculated from eq. 10, derived from eqs. 3 and 5.

$$P = \left(\frac{C_M}{C_S - A \sum_{j=1}^{j=3} \varkappa_j^{-1} j h^j} - 1 \right) h K_{HA}^{-1} \tag{10}$$

A in eq. 10 was obtained by transforming eqs. 3 and 4 to

$$A = \frac{C_A - C_M}{\sum_{j=1}^{j=3} \varkappa_j^{-1} h^j} \tag{11}$$

The constants b_1 and b_{MHA} were then computed from eq. 3 after previous calculation of the variables h, M and A. Figures for M and MOH were included in these calculations. Hydrolysis of Fe(III) ions (27, 28) was not considered earlier, due to the apparent strong Fe(III) complexes formed by PS. The complex, MOH, was thought to be the single Fe(III) hydroxy complex, to be formed at the experimental conditions employed (28). The value 2.62×10^{-3} for b_{MOH}, obtained by Milburn *et al.* (28) at 25° and 0.15 ionic strength, was used. Values of M and A were determined graphically from eqs. 10a and 11a, derived from the same equations as those indicated.

$$A(P + h K_{HA}^{-1}) \sum_{j=1}^{j=3} \varkappa_j^{-1} j h^j - M[h K_{HA}^{-1} + b_{MOH}(P h^{-1} + 2 K_{HA}^{-1})] = C_S(h K_{HA}^{-1} + P) - C_M h K_{HA}^{-1} \tag{10a}$$

$$A \sum_{j=1}^{j=3} \varkappa_j^{-1} h^j - M(b_{MOH} h^{-1} + 1) = C_A - C_M \tag{11a}$$

In respect of the above equations the plot $M(A)_{C_M, C_A, C_S, h}$ for each experimental measurement resulted in two straight lines, whose point of intersection furnished the actual values of M and A. The equations were constructed from their intercepts.

The results of the 1:7 (C_M:C_A) titration are listed in Table 4. The logarithms of the stability constants calculated from both the 1:7 and 1:4 titrations are compiled in Table 3. The value of P, obtained at the 1:7 titration, was also used in the calculations of the 1:4 titration.

Table 4. Computation of the apparent stability constants (in M^{-1}) for the Fe(III) complexes at 25°C and 0.15 ionic strength. The numbers in parentheses in the table heads refer to equation numbers.

$C_A = 2.06 \times 10^{-3}$, $C_M = 3.00 \times 10^{-4}$ (initial concentrations).

ml KOH (0.1028 M)	$h \times 10^{-7}$	$A \times 10^{-7}$ (5)	Z (6)	$b_2 \times 10^7$ (7 c)
0.735	21.98	0.709	1.560	1.80
0.765	15.92	1.122	1.64	1.58
0.800	11.10	1.808	1.73	1.51
0.833	7.245	3.08	1.82	1.50
0.870	3.802	6.50	1.915	1.66
				Av. 1.6

ml KOH	$h \times 10^{-4}$	$A \times 10^{-12}$ (11)	$Z = 1.0$	$P \times 10^7$ (10)
0.050	19.95	0.156		2.55
0.150	15.63	0.264		1.85
0.250	11.06	0.551		2.10
0.350	6.607	1.610		1.75
0.453	2.344	13.4		1.17
0.503	0.8512	101		2.49
				Av. 2.0

$C_A = 2.06 \times 10^{-3}$, $C_M = 3.03 \times 10^{-4}$ (initial concentrations)

ml HCl (0.1010 M)	$h \times 10^{-3}$	$A \times 10^{-14}$ (10a, 11a)	$M \times 10^{-5}$ (10a, 11a)	$b_1 \times 10^{14}$ (3)
0.103	2.203	8.54	1.0	2.0
0.203	2.630	6.87	1.7	1.5
0.303	3.020	4.64	2.0	1.5
0.400	3.443	3.58	2.5	1.5
0.500	3.846	2.83	2.95	1.5
0.700	5.105	1.84	3.8	1.5
0.800	5.508	1.49	5.0	1.2
				Av. 1.5

$b_{MHA} = 7.2 \times 10^6$

The error in the constants is considerable as apparent from the discrepancies between the constants computed from the two different titrations. At low pH, the individual calculations (Table 4) do not reasonably indicate true error in the constants (cf. 24); e.g. assuming an error of ± 0.005 in pH at the 1:7 titration the corresponding error in log b_1 will be within ± 0.46 log units at delivery of 0.500 ml HCl.

The Mn(II), Mg(II) and Ca(II) complexes

Complex formation is apparently indicated in Figs. 2 and 3 by deflections of the metal titration curves from the ligand curves at the ionizations of H_2A and HA. All

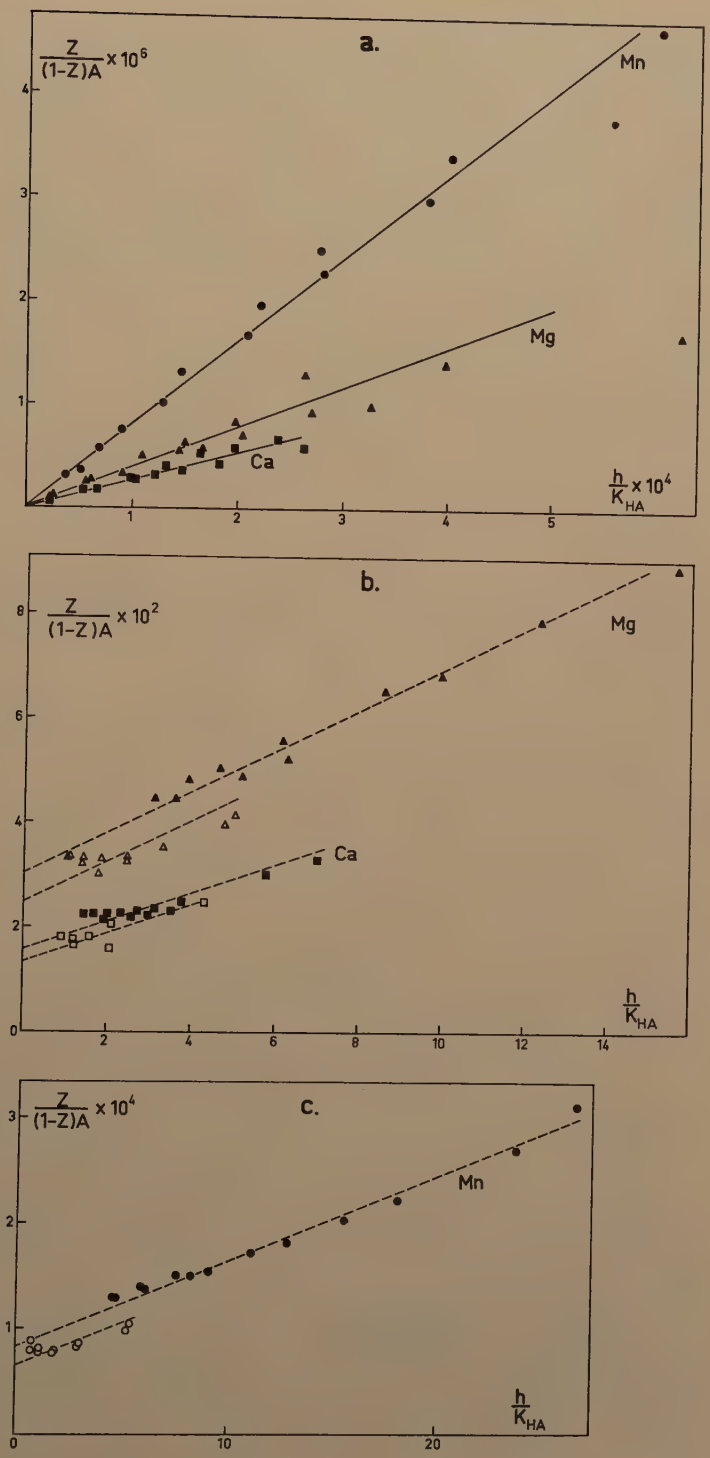


Fig. 4. Survey of the computation of b_{MHA} (a) and b_1 (b and c) from the Ca(II), Mg(II) and Mn(II) titrations. Experimental data plotted by means of eq. 7a (see the text). The lines are drawn from the values of the constants in Table 3, using eq. 7a.

curves are inflected at a $-\log h$ value equal to the inflection region at the end of the ionization of H_2A. Most of the curves also confluence with the ligand curve in this region. This may indicate that MHA species are formed. The formation of a further complex, MA, for these metals was established at the calculation of the stability constants. The logarithms for the stability constants of the complexes mentioned are listed in Table 3. Log b_{MHA} is only given for the 9:1 titrations, as in the range for the formation of MHA species, the 1:2 titrations resulted in too low pH decrements to make any accurate calculations possible. Formation of M_2A species was not considered at the computation of b_1. Results of the individual calculations are surveyed graphically in Fig. 4, using eq. 7a. After dividing this equation with $(1-Z)A$ the resulting left hand side, $Z(1-Z)^{-1}A^{-1}$ has been plotted against hK_{HA}^{-1}, giving the slope b_{MHA} and the intercept zero (Fig. 4a) or b_1 (Figs. 4b and 4c).

The distribution of the plotted points in Fig. 4 (b and c) indicates that the slight differencies in intercepts between the 9:1 and 1:2 titration lines apparently are within experimental uncertainty (cf. Table 3). The slight vertical displacement of the 9:1 titration plots from those of the 1:2 titrations may, however, be due to formation of M_2A complexes at the former titrations. The intercepts for the 9:1 titration lines will then be equal to $b_1 + b_1 b_{M_2A} \frac{1-2Z}{1-Z} M$ instead of b_1. If this is true, $\log b_{M_2A}$ will be: calcium(II) 0.99; magnesium(II), 1.13 and manganese(II), 1.22.

The upward deviation of the plots (most marked at high C_A) in Fig. 4 (b and c) for decreasing h, is probably due to change in activity conditions and error in b_{MHA}.

Discussion

Recent studies of apparent ionization constants of serine (29–31) and organic phosphoryl compounds (29, 32–35) may conclude that pK_{H_3A}, pK_{H_2A} and pK_{HA} (Table 2) are related to the carboxyl, secondary phosphoryl and amino ionizations of PS, and that the primary phosphoryl group of the ligand ionizates at a pH below 2.

Albert (30) and Li *et al.* (31) reported values of 14.60 and 14.54 respectively for log β_2 of the copper(II) serine complex. These values approach the value 15.51 obtained for log β_2 of the corresponding Cu(II) PS complex. The difference in value of log β_2 may be due to the presence of the phosphate group in the PS complex. No sure evidence for complex formation between serine and Fe(III) ions was attained in the investigation made by Albert (30). The Fe(III) PS complexes reported in the present study may, thus, mainly involve the phosphate group of the ligand. Qualitative data on the association of ferric ions to orthophosphate also confirm this suggestion (7, p. 31). Apparently, it is only the ligand phosphate group, which also coordinates the metals, Ca(II) and Mg(II), in the MHA complexes. The values of b_{MHA} for these complexes agree within experimental uncertainty with stability constants given for complexes between the same metals and adenosinemonophosphate (34, 36), glycerophosphate and fructosphosphate (35). The investigations referred to were carried out at the ionic strength 0.1 and 0.2, and the phosphate ester group only was found to be responsible for the complex formation (35). Other investigations in this field have been reviewed by Smith *et al.* (36).

The decreasing order of complex-forming ability of the investigated metals was found to be Fe(III) > Cu(II) > Mn(II) > Mg(II) > Ca(II). This order was also related to the order of decreasing chelating ability by Longenecker *et al.* (8) in the study

of the catalytic ability of different metals. The order of catalytic ability was found to be the same as that of the chelating ability of the metals mentioned, except in the case of Fe(III) and Cu(II). For the latter metals, the order was the reverse. Data for Ca(II) were not included in that investigation.

From the computed values of the constants K_{HA}, b_1, and b_{MHA} the pK-values of the acid complexes, MHA, were calculated (36). The results has been compiled in Table 3.

The present study, performed at an ionic strength (=0.15) similar to the physiological, indicates that PS at physiological pH may form soluble and very stable 1:2 (metal:ligand) complexes with Cu(II) and Fe(III), when $C_A/C_M = 2$ and $C_A \approx 10^{-3}$ M.[1] The Fe(III) complex formed under these conditions is soluble in contrast to the corresponding 1:1 complex, formed at lower pH. Moreover, it may be noted that formation of the MA-complexes of PS and the metals Mg(II) and Ca(II), the most stable complexes investigated for these metals, [as well-known Ca(II) and Mg(II) are involved in bone formation, and activation of some phosphatases (5,37)] occurs at a pH-range (cf. pK_{MHA} in Table 3) similar to the region of pH optimum for alcaline phosphatases (37), as, for example phosphomonoesterase of bone.

SUMMARY

Apparent acid ionization constants and apparent stability constants of iron(III), copper(II), manganese(II), magnesium(II) and calcium(II) complexes of O-phosphorylserine have been determined potentiometrically at 25°C and 0.15 ionic strength. Possible composition of these complexes have been suggested. The relative values of the stability constants were found to be iron(III) > copper(II) > manganese(II) > magnesium(II) > calcium (II). The ionization constants of the acid complexes of phosphorylserine with these metals have been computed.

I wish to express my sincere thanks to Docent Ido Leden for valuable discussions and criticism. The investigation was aided by a grant from the *Medical Faculty, University of Gothenburg.*

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[1] In fact it may be calculated from the constants computed (Table 3), using eqs. 7a and 6 that the MA_2 complex of Cu(II) will dominate ($Z \geq 1.5$) at pH 7.4 down to $C_A = 5.4 \times 10^{-4}$, when $C_A/C_M = 2$, and to $C_A = 1.8 \times 10^{-4}$, when $C_A/C_M = 10$. Assuming the MA_2 complex to be formed similar figures for Fe(III), when $Z \geq 1.9$, will be $C_A = 1.2 \times 10^{-3}$, when $C_A/C_M = 2$, and $C_A = 7.5 \times 10^{-5}$, when $C_A/C_M = 10$.

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A phosphopeptide containing histidine obtained from cow's casein by partial hydrolysis with proteolytic enzymes

By Ragnar Österberg

With 3 figures in the text

Partial hydrolysis of casein with proteolytic enzymes yields a phosphopeptide-containing product, which can be separated from the other products of hydrolysis. Numerous preparations of this phosphopeptide fraction have been made, as apparent from recent reviews of the literature (1–3). All these preparations, however, resulted in products, which presumably formed a mixture of different phosphopeptides (4, 5). Using phosphopeptide fractions, obtained by digestion of casein with pepsin followed by pancreatin, Mellander *et al.* (4, 6–9) found that these compounds could be used to promote the intestinal resorption of calcium and iron. Calcium in this form was also well utilized in bone calcification of growing animals (6, 7) and rachitic children (4).

In order to elucidate the chemical nature of the interaction between metals and phosphopeptides, a synthetic model substance of similar structure has been investigated in respect of metal binding properties (10, 11). It is, however, clear that this interaction cannot be fully understood until the structure of the casein phosphopeptides mentioned—especially as far as the phosphorus bonds are concerned—is better known. The present study describes the isolation, composition and acid-base titration of a pure phosphopeptide. This peptide was separated by ion-exchange chromatography from a phosphopeptide mixture, prepared from an enzymic hydrolysate of cow's casein, mainly as described by Mellander (1).

Materials and Methods[1]

Casein was prepared from fresh cow's milk by isoelectrical precipitation and was rinsed once with water. *The enzymes* used for digestion of the casein, i.e. pepsin (1:10,000), pancreatin and crystalline trypsin, were commercial products (Parke, Davies & Co. and Armour & Co., respectively) and were used without further purification. No alcaline phosphatase activity could be detected in the pancreatin, when monophenylphosphate was used as a substrate.

[1] The preparations of the digested and re-digested phosphopeptide mixtures were completed in collaboration with Dr. H. Bennich and Dr. B. Johansson of this institute.

Enzymic digestion of the casein

Wet casein, corresponding to about 8 kg dry weight, was dispersed in 70 l. aq. dest. and acidified under vigorous stirring to pH 1.7 by diluted hydrochloric acid. This suspension was then transferred to a 100 l glass vessel. The casein was first digested with pepsin for seven days at 37° ± 0.5°C. 30 g pepsin, dissolved in 300 ml 0.01 *M* HCl, was added to the suspension, and this was repeated on the third and fifth day. The break-down of the casein was followed by formol titration according to Mellander (1). From the fifth day, no more change in formol-titration value could be detected.—On the eighth day, the pH of the digested solution was brought to 7.5 by adding dilute NaOH. 50 g pancreatin in a 5 per cent water solution was added and digestion allowed to proceed for 24 hours, when a further 50 g pancreatin was added. After another 24 hours of digestion, 50 g pancreatin was again added. The digestion was continuously followed by formoltitration, and constant values were obtained after 50 hours. At the beginning of the fifth day of the pancreatin digestion, the temperature was reduced to about 13° and pH adjusted to 4.6 by adding glacial acetic acid. Insoluble substances were allowed to settle for 24 hours, after which the supernatant was treated with saturated lead acetate. The precipitate thus obtained, was suspended in about 10 l aq. dest., and the lead removed by treatment with H_2S. The precipitated PbS was washed once with aq. dest., after which H_2S was removed from the combined supernatants by airation, and the solution then taken up to a concentration of 70 % of ethanol by adding 96 % ethanol. The resultant precipitate was washed with ethanol and ether, and then dried *in vacuo* for 6 days at 60°C. Yield: 230 g. Analyses:[1] N, 12.4; P, 4.4; (inorganic P < 0.1) Na, 2.5 per cent; N/P-ratio, 6.3.

During the entire digestion procedure the pH was adjusted every day at 1.7–2.0 and 7.5–8.5 respectively by adding HCl or NaOH at the peptic and tryptic digestions. Daily additions of about 50 ml toloul and 100 ml chloroform were used as bacteriostathics.

The resultant product was then re-digested with crystalline trypsin. This digestion was performed at pH 8.3 and 37° for four days. At the beginning of the first two days, 0.5 g trypsin suspended in 10 ml aq. dest. was added to the digestion solution of 2 l containing 50 g of the previously digested product. No significant change in pH or formol-titration value appeared in the course of digestion, and at the beginning of the fifth day, the digestion was interrupted. A phosphopeptide fraction was then prepared in the same way as described above, and after final drying at 60° *in vacuo* for two weeks, the weight was found to be 4.6 g.

Ion-exchange chromatography on Dowex 50-X2

The resin, 200–400 mesh, was used in the hydrogen form. Before the separation the resin was rinsed by treatment with *M* NaOH and 4 *M* HCl. The dimensions of the column was 50 × 1.6 cm, and several litres of 0.01 *M* HCl, used as influent, were allowed to pass before the sample was added. About 300 mg of the re-digested fraction was separated on each occasion. It was added to the column dissolved in

[1] The analyses of N and P in the present study were performed by Mikroanalyslaboratoriet, Medicinsk-Kemiska Institutionen, Uppsala. Inorganic P was analysed according to Martin and Doty (12), Na was determined by flamephotometry.

about 10 ml aq. dest., to which sufficient 0.1 *M* NaOH had been added in order to get all phosphopeptide into the solution. The eluation was performed at a rate of flow of about 40 ml per hour. Fractions of 4 ml were collected. The location of the phosphopeptides was determined by the photometric ninhydrin method developed by Moore and Stein (13), directly and after alcaline hydrolysis (14) on 0.10 and 0.05 ml aliquots, respectively. The eluates of the middle of each peak were collected and evaporated *in vacuo* until reaching about one fifth of the volume, the temperature never exceeding 20°. The phosphopeptides were then precipitated from the evaporated solution by adding ethanol and ether. The resultant precipitate was then washed with 99.5 % ethanol and ether (distilled over sodium) and was left to dry *in vacuo* for one week at 60° over P_2O_5.

Paper electrophoresis

This was carried out in a LKB-paper electrophoresis unit at 400 V and pH 5.0 or 2.8 for three hours in a 0.05 *M* NaAc/HAc buffer or 0.2 *M* HAc, respectively at ambient temperature (about 22°C).

About 0.1–0.3 mg substance was applied to the paper in all the runs. Schleicher and Schüll papers 2043 B, 4 × 41 cm were used. The phosphopeptides were made visible on the paper by the developing technique described by Reindel and Hoppe (15).

Determination of constituent amino acids

The phosphopeptides were hydrolysed in sealed tubes at 110°, using 6 *M* HCl. For qualitative analyses the hydrolyses were continued for 22 hours. The resulting hydrolysates were then subjected to a twodimensional paper chromatography on Whatman No. 1 paper, using butanol/glacial acetic acid/water (v/v/v/ = 4/1/5) in the first, and phenol/water (v/v = 20/80) in the second direction.

For quantitative determinations, the hydrolyses were continued for 22, 72 and 144 hours and the hydrolysates were analysed by the method developed by Moore and Stein[1] (16) or by the dinitrophenylation (DNP) method of Sanger (17) as described by Koch and Weidel (18). The analyses by these two methods were performed on 3 mg and 1.5 mg hydrolysed phosphopeptide, respectively.—In order to find out, whether isoleucine or leucine was present in the analysed phosphopeptide, one millimole of leucine was added to one of the hydrolysates, which were analysed according to Moore and Stein.—The DNP amino acids were identified by chromatography on a phthalate buffered Whatman No. 1 paper, using the phthalate/*tert*-amyl alcohol solvent of Blackburn and Lowther (19). No data for basic amino acids are to be reported by the DNP-method, in view of the fact that the ether-soluble extract of the DNP amino acids only was analysed.

N-terminal group analysis

Sanger's DNP-technique as described by Levy *et al.* (20) was used. 1–2 mg of the phosphopeptide fraction was allowed to react with fluorodinitrobenzene dissolved

[1] The author is indebted to Mr. L. Strid for these analyses.

in aqueous ethanol, containing sodium bicarbonate. The DNP-peptides were hydrolysed by 6 *M* hydrochloric acid for 10 hours, and the resulting DNP-amino acids were identified by paper chromatography as described above.

Potentiometric titration

The titrations were carried out at 25°C and 0.15 ionic strength with the use of the same titration apparatus as described by the present author (11). Carbonate-free 0.0618 *M* KOH was prepared in a manner similar to that described by Powell and Hiller (21), and was standardized against potassium hydrogen phthalate. 0.0959 *M* HCl was prepared from constant boiling hydrochloric acid and was standardized against potassium iodate. Acid or base was added from an "agla" micrometer syringe (Burroughs Welcome & Co., London). The syringe was attached to a glass capillary, dipped into the titration solution after each addition of acid or base. The phosphopeptide was dissolved in 20.00 ml 0.15 *M* KCl, to which solution potassium hydroxide was added up to pH 10.5. This solution was then titrated with hydrochloric acid to pH 2.5, the pH being recorded at each acid delivery. The quantities of HCl added were corrected from a blank titration, performed in the same manner. Carbon dioxide was kept out from the titration vessel by bubbling nitrogen, which previously had passed through a 10% sodium hydroxide solution and an 0.15 *M* KCl solution, immersed in the thermostat and rinsed from droplets by a cotton wool filter. The standardization of the pH-meter and the convertion of recorded pH values to values of hydrogen concentration were carried out as described in (11).

Results

Chromatography of the re-digested fraction on Dowex 50–X2 produced the effluent curve, shown in Fig. 1, where the data of paper electrophoresis are also indicated. If the chromatographic solvent was changed from dilute hydrochloric acid to an acetate buffer, it was observed that two more ninhydrin positive peaks could be detected. These fractions have not, however, been analysed yet. The eluates corresponding to the peaks of Fig. 1, were collected separately according to the vertical lines. Fractions I and II of this chromatography yielded 115 mg and 8.5 mg respectively. A pooled fraction from three such chromatographic runs was used to obtain the data of fraction II, stated below.

The re-digested phosphopeptide mixture, fractions I and II, were analysed for nitrogen and phosphorus, and the results are shown in Table 1. Qualitative amino acid analyses and N-terminal residues of the same fractions are compiled in Table 2.

Table 1. Nitrogen and phosphorus contents of the re-digested fraction (R), fraction I (I) and fraction II (II).

Fraction	% N	% P	N/P (molar ratio)
R	11.9	5.2	5.0
I	11.3	5.55	4.5
II	11.0	5.9	4.15

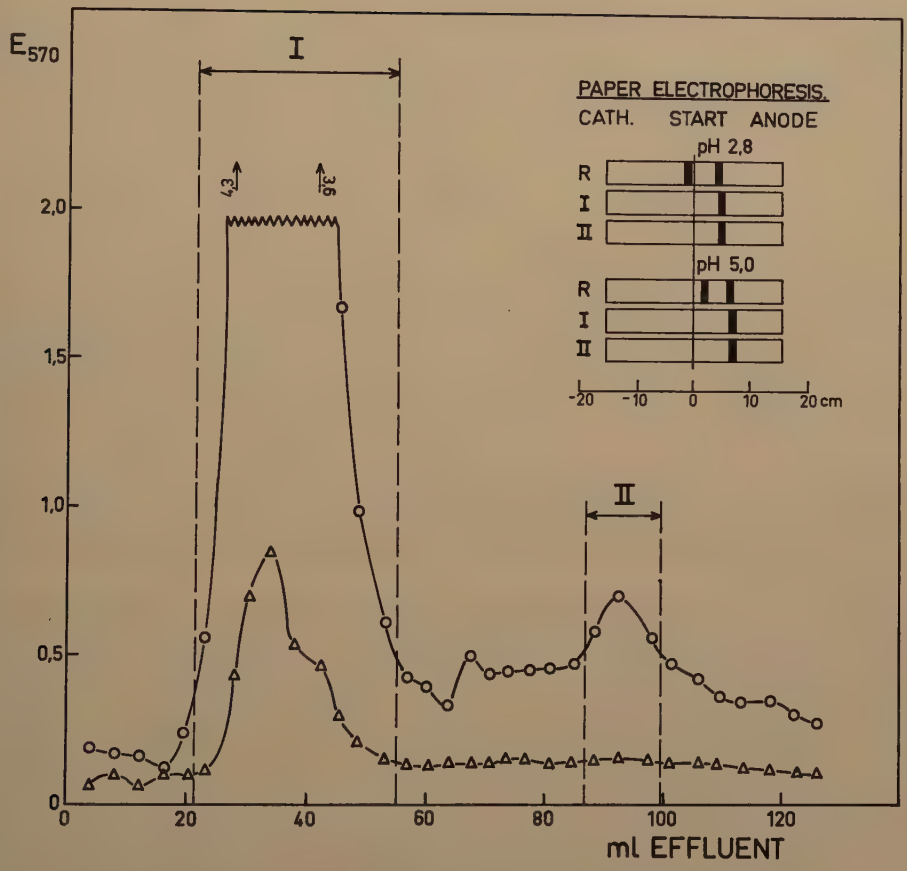


Fig. 1. Chromatography of 310 mg re-digested phosphopeptide mixture (R) on Dowex 50–X2, using 0.01 *M* HCl as influent. Ninhydrin analyses on 0.10 and 0.050 ml aliquots were carried out directly (△) and subsequent to alcaline hydrolysis (○). Paper electrophoretic runs were performed in 0.05 *M* NaAc/HAc buffer, pH 5.0, or 0.2 *M* HAc, pH 2.8, for three hours at 400 V, using 4 × 41 cm Schleicher & Schüll papers.

Table 2. Qualitative amino acid composition and N-terminal residues of the re-digested fraction (R), fraction I (I) and fraction II (II). Faint spots are indicated by brackets.[1]

	Amino acids found after 22 hours of hydrolysis											N-terminal residues					
Fraction	Ala	Prol	Ser	Gly	Thr	His	Lys	Glu	Asp	Ileu Leu	Val	Ser	Ala	Glu	Asp	Ileu Leu	Val
R	+	+	+	(+)	+	(+)	+	+	+	+	+	(+)	+	+	+	+	+
I	+	–	+	–	+	–	–	+	+	+	+	–	–	(+)	+	+	–
II	–	–	+	–	(+)	+	–	+	–	+	+	–	–	+	–	–	–

[1] Abbreviations of the amino acids according to Brand and Edsall (34).

Table 3. Quantitative amino acid composition of fraction II analysed by the method of Moore and Stein (St. and M) and according to the method of Koch and Weidel (K and W). The values are corrected for blank, and they are expressed in molar ratios, based on the minimum glutamic acid content taken as 3.00.

Method	Duration of hydrolysis	Molar ratios of amino acids					
		Ser.	Thr.	His.	Glu.	Ileuc.	Val.
St. and M	22 hours	2.75	0.27	0.79	3.00	1.00	0.89
St. and M	72 hours	2.05	0.78	0.95	3.00	1.60	1.03
K and W	144 hours	1.38	0.61	—	3.00	1.82	0.97
Probable minimum number of amino acid residues		3.0	1.0	1.0	3.0	2.0	1.0

All DNP-amino acids, related to the N-terminals given in Table 2 were detected only in the ether-soluble extracts of the hydrolysed DNP peptides. All water extracts were colourless, except the one related to the redigested fraction, where ε-DNP lysine was identified.

Fraction II was further investigated by quantitative amino acid analysis, and the results are listed in Table 3. The minimum number of 3 residues recorded for serine in this table was obtained by extrapolating the values of molar ratio to zero time (resulting in the molar ratio value 3.08). Molar ratio was plotted against time, and the plotted points fitted to a curve. The amount of desctruction of serine follows a course similar to that reported for destruction of serine in other phosphopeptides (22).—The marked increase in molar ratio for threonine, following upon the hydrolysis being extended from 22 to 72 hours, seems confusing. However, threonine in the present phosphopeptide may be adjacent to (an) amino acid(s) with stabilizing effects on the peptide bond(s) (23). Table 3 indicates this (these) amino acid(s) to be isoleucine, since it shows a similar increase in molar ratio as threonine. This is also in accord with the known resistance of isoleucine peptides to acid hydrolysis (23, 24).

Ion-exchange chromatography, paper electrophoresis, N-terminal group analysis and quantitative amino acid analysis of fraction II do not contradict that this fraction constitutes a single peptide. The quantitative amino acid analysis made it possible to calculate a minimum molecular weight for this phosphopeptide. The minimum molecular formula $C_{51}H_{86}N_{13}O_{31}P_3$ was applied, and the minimum molecular weight was found to be 1470. A phosphopeptide of this composition would have a nitrogen content of 12.4 %, a phosphorus content of 6.3 %, and a N/P ratio of 4.3. This seems to be reasonably compatable with the analytical data shown in Table 1, as no correction for ash content was carried out in report of these data.

Potentiometric titration

To gain further information of composition and structure of the fraction II peptide, acid-base equilibria of this peptide were studied by potentiometric tiration.

This titration furnished the titration data plotted in Fig. 2. These data indicate

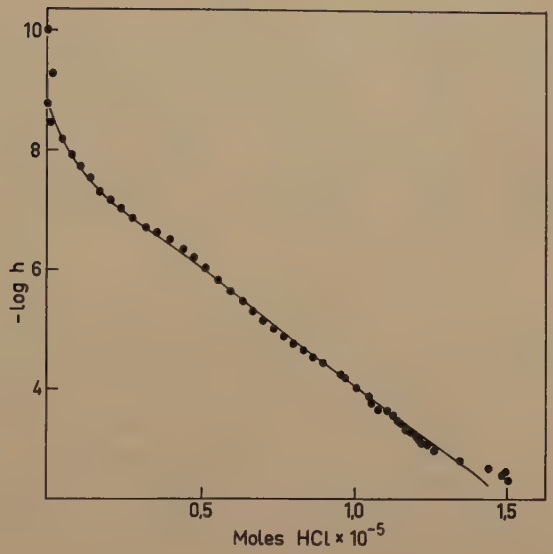


Fig. 2. Titration of fraction II at 25°C and 0.15 ionic strength, corrected for blank (●). 2.599 mg phosphopeptide dissolved in 20.00 ml 0.15 *M* KCl to pH 10.46 by the addition of 0.399 ml 0.0618 *M* KOH and titrated with 0.0959 *M* HCl. The drawn curve has been calculated from the constants in Table 4.

a marked buffering action of the peptide in the region for ionization of secondary phosphoryl groups (around pH 6). On computation of the ionization constants it was therefore assumed that all the phosphorus in the phosphopeptide was bound through monoester linkages to hydroxyl amino acids. (Other probable phosphate linkages as diesters of ortho- and pyrophosphoric acid include no secondary phosphoryl groups.) The minimum molecular composition of this peptide will then include 12 titratable groups; corresponding to the first and second ionizations of three phosphate groups, the ionization of a C-terminal carboxyl group, three glutamic acid carboxyl groups, an imidazole—and an N-terminal amino group. Only nine acid groups were in fact considered to be titrated in this study, however, as primary phosphoryl groups usually ionizate below pH 2 (11, 25 pp. 133–134).

The total uptake of protons for 2.599 mg phosphopeptide was found to be equal to 1.500×10^{-5} moles of HCl. If these moles thus correspond to nine acid groups, 0.500×10^{-5} moles of HCl may have been consumed at the deionizations of three secondary phosphoryl groups. This agrees with the value of 0.494×10^{-5} moles of HCl calculated as required for the amount of phosphorus present.

Table 4. Apparent ionization constants of the fraction II peptide. The computations were made graphically, by means of eqs. 5–7.

r	*t*	pK_{HA}	pK_{H_2A}	pK_{H_3A}	pK_{H_4A}	pK_{H_5A}	pK_{H_6A}	pK_{H_7A}	pK_{H_8A}	pK_{H_9A}
0	3	7.69	6.84						3.07	2.66
2	5			6.36	5.67		4.38	3.87		
1	7		6.94	6.42	5.68	5.12				
4	4					4.93	4.33	3.70	3.12	
Average		7.7	6.9	6.4	5.7	5.0	4.35	3.8	3.1	2.65

Computation of apparent ionization constants of the phosphopeptide titrated was carried out by an adaption of the graphical method outlined by Scatchard (26). In this way the pK-values related to the nine overlapping ionizations could be computed without a great deal of labour and with an error probably not exceeding ± 0.10 pK units (cf. 26). This degree of accuracy seemed sufficient, in view of the fact that up to ten per cent of the titrated substance might consist of impuritites. The computed constants are listed in Table 4. They were obtained in the manner, now to be summarized below.

The following symbols will be used:

A	Completely ionized form of a polybasic acid H_jA
C_A	Molar concentration of all A species
C_S	Molar concentration of bound protons; equal to the molar concentration of total HCl added, corrected for blank
h	Molar concentration of free hydrogen ions
j	Number of protons bound in the acid H_jA
J	Maximum value of j
K_{H_jA}	Apparent ionization constant of the acid H_jA, defined by $K_{H_jA} = h[H_{j-1}A][H_jA]^{-1}$
$\bar{n}_H$	Average number of protons bound per C_A
r, t	Integers, being $\geqslant 0$ and $\leqslant J-1$; $1 \leqslant J-t-r \leqslant J$
P	$= J-t$
$\varkappa_j$	$= \prod_0^j K_{H_jA}$; $\varkappa_0 = 1$.
[ ]	Molar concentration of the species indicated.

A polybasic acid H_jA may successively ionizate to result in the species $H_{J-1}A$, $H_{J-2}A$, $H_{J-3}A \ldots A$. If all these species are considered as existing, C_A and C_S are given by

$$C_A = \sum_{j=0}^{J} [H_jA] \tag{1}$$

$$C_S = \sum_{j=1}^{J} j\,[H_jA] \tag{2}$$

The relation between C_A and C_S is

$$C_S = \bar{n}_H\, C_A. \tag{3}$$

For computation of ionization constants related to the J different ionization steps of H_jA, the experimental titration range may be divided into parts. Each such part may be limited by $r < \bar{n}_H < P$. All different species of A included in any such range of $\bar{n}_H$, may be presumed to be H_PA, $H_{P-1}A \ldots H_rA$. The average number of protons taken up within each range per all A species assumed to be present, $\bar{n}_H - r$, is then given by

$$\bar{n}_H - r = \frac{\sum_{j=r+1}^{j=P} (j-r)\,[H_jA]}{\sum_{j=r}^{j=P} [H_jA]} \tag{4}$$

Using this equation, a function Y may be defined by the relation

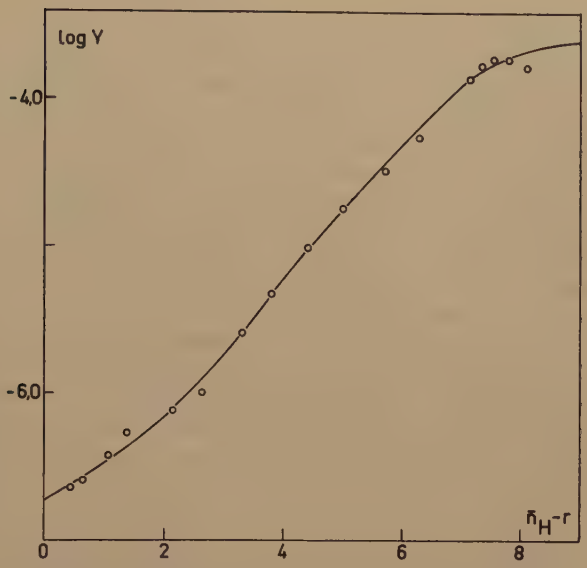


Fig. 3. Computation of apparent ionization constants of the fraction II peptide. Experimental data plotted by means of eq. 5 as the curve log Y $(\bar{n}_H - r)$ for $r = 0$ and $t = 3$. Intercepts and limiting slopes of the curve correspond to the constants in Table 4.

$$Y = h\left(\frac{P-r}{\bar{n}_H - r} - 1\right) = \frac{\sum_{j=r}^{j=P-1} \varkappa_j^{-1}(P-j)\,h^{j-r}}{\sum_{j=r+1}^{j=P} \varkappa_j^{-1}(j-r)\,h^{j-r-1}} \cdot \tag{5}$$

The function Y, being similar to the Scatchard function Q (26), may be used for computation of ionization constants related to any range of $\bar{n}_H$. The constants will be the only unknown quantities of eq. 5. The values of $\bar{n}_H$ and Y may be calculated for each specimen of experimental measurement from data on C_A, h and C_S, using eqs. 3 and 5. Graphical computation of the constants according to Scatchard may thus be performed.

The limiting values of Y in eq. 5 when h approaches zero and infinity are $K_{H_{r+1}A}(P-r)$ and $K_{H_PA}(P-r)^{-1}$, respectively. Two constants may thus be computed by extrapolating log Y of the curve log $Y(\bar{n}_H - r)$ to the abscissa values zero and $P-r$ (cf. Fig. 3).

Two further constants can be obtained from the limiting slopes of the curve log $Y(\bar{n}_H - r)$, when $\bar{n}_H - r$ approaches the above-mentioned values. Hence, as $\bar{n}_H - r$ and h approach zero, the derivative of log Y with respect to $\bar{n}_H - r$ is given by

$$\lim_{(\bar{n}_H - r)\to 0} \frac{d \log Y}{d(\bar{n}_H - r)} = \left(\frac{P-r-1}{P-r} - \frac{2K_{H_{r+1}A}}{K_{H_{r+2}A}}\right) \log e \tag{6}$$

and as $\bar{n}_H - r$ approaches $P-r$ and h^{-1} approaches zero

$$\lim_{(\bar{n}_H - r)\to P-r} \frac{d \log Y}{d(\bar{n}_H - r)} = \left(\frac{P-r-1}{P-r} - \frac{2K_{H_{P-1}A}}{K_{H_PA}}\right) \log e \tag{7}$$

A single plot log $Y(\bar{n}_H - r)$ may, thus give four constants. By appropriate selection of r and t, a sufficient number of plots may be performed to compute all the J constants.

In the present study some extrapolations of the plots log $Y(\bar{n}_H - r)$ were subjected to significant experimental errors due to some scatter in the data (cf. 26). Each intermediate constant was therefore computed from two diffferent plots. This computation was started by the calculation of two temporary values for each of these constants from preliminary values of intercepts or limiting slopes. The temporary values were then successively adjusted to give most satisfactory agreement relative each other by considering, within the range of experimental error, other possible courses for the extrapolated curves. On each occasion this adjustment had to run successively through all the preliminary values, due to the nature of the overlapping for the plots. The figures in Table 4 are the result of two such successive approximations.

From the computed constants a titration curve was calculated, the result is shown in Fig. 2. The discrepancies between the experimental data and the calculated curve at low $-\log h$ may be explained by slight uptake of protons by primary phosphoryl groups or the great experimental error caused by the blank correction in this part of the titration curve. (The amount of HCl added to the blank and peptide solutions respectively became here figures of the same size.)

Discussion

Acid partial hydrolysates of phosphoproteins have recently (22, 27–30) been separated by ion exchange chromatography in a way similar to that described above. The phosphate groups of those phosphopeptides are usually assigned to be linked through monoesters to hydroxyl groups of serine or threonine; some of these linkages, however, may be the result of the acid hydrolysis (3, 31). The constitutent amino acids of casein phosphopeptides isolated in that manner (22, 30) are all to be found in the present re-digested fraction (Table 2).

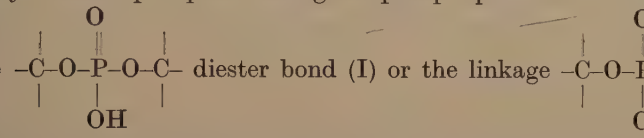
In respect of qualitative amino acid composition, this fraction also agrees with previous phosphopeptide mixtures, prepared from enzymic digests of casein (5, 32). Recent quantitative amino acid analyses (1–3, 5, 9) have shown glutamic acid, serine and isoleucine to constitute the main amino acids of these peptide mixtures. The same main amino acids constitute the pure phosphopeptide of this study (Table 3). This phosphopeptide, however, differs from earlier investigations in that it contains histidine at an amount sufficient to be considered as residue.

The content of hydroxyl amino acids and phosphorus in phosphopeptides from enzymic hydrolysates (1–3, 5, 9) (Tables 1, 3) allow all the phosphorus to be bound as in phosphopeptides from acid partial hydrolysates. This type of phosphate linkage was assumed to occur in the present fraction II phosphopeptide, when its apparent ionization constants were computed. The number of acid groups titrated was then found to agree with the number of groups calculated to be titratable between pH 10.5–2.5 from amino acid composition and phosphorus content.

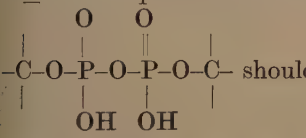
The apparent pK-values computed (Table 4) may roughly be used for checking the identity of the peptide acid groups. The values of pK_{HA} (7.7) and pK_{H_2A} (6.9) may belong to the glutamic acid N-terminal amino group and the histidine imidazole group of the peptide, respectively. In dipeptides these groups have been reported (25) to have pK-values of 7.62, 7.52, 7.75 and 6.82, 6.92, respectively; the histidine-peptides containing acid amino acids. The next three pK-values listed in Table 4 ($pK_{H_3A} - pK_{H_2A}$) have a mean value of 5.7. They may therefore be attributed to the last ionization of three nearly identical monoesterbound phosphate groups,

as the same ionization in O-phosphorylserine gives the pK-value 5.66 (11). $pK_{H_4A} - pK_{H_3A}$ (4.35, 3.8, 3.1) and pK_{H_2A} (2.65) have the values of pK, which can be expected to belong to the three glutamic acid carboxyl groups and the C-terminal carboxyl group of the present peptide, respectively (25). Any primary phosphoryl groups were not thought to need consideration in the present titration, as these groups usually are not titratable above pH 2 (11, 25 pp. 133–134). The linkages of the peptide phosphate groups, in fact, could not be readily explained either, if one or more primary phosphoryl groups were wholly titrated.—The presence of such acid groups in the peptide may, however, be apparent from the paper-electrophoretic runs (Fig. 1). When pH was changed from 5.0 to 2.8 at these runs, there appeared to be a slight decrease only of the distance moved. At both pH, the phosphopeptide had probably a distinct negative net charge, since it evidently moved to the anode.

Investigations made by Perlmann (3) have provided evidence of the presence of many different phosphate linkages in phosphoproteins and compounds related thereto.



The –C–O–P–O–C– diester bond (I) or the linkage –C–O–P–N– (II) probably does not constitute phosphate linkages in the present peptide, nor does a phosphate-imidazole linkage (III) (31, 33). If any of these linkages existed in the phosphopeptide, eight (I, III) or seven (II) protons only should have been taken up to pH 2.5, and only three protonations of the peptide should probably have occurred at pH 6 ± 1. The presence of a pyrophosphate group, linked as proposed by Perlmann,



–C–O–P–O–P–O–C– should have furnished even more different titration data. Then two protons only should have been taken up at pH 6 ± 1.—Accordingly, the monoester bond, assumed to be present all through this paper, will be left as the most probable linkage for all the phosphate groups of the fraction II peptide.

SUMMARY

A pure phosphopeptide has been prepared from an enzymic cow casein hydrolysate, obtained by treating the casein with pepsin, followed by pancreatin and crystalline trypsin. The phosphopeptide was found to have three phosphate groups and the following amino acid composition: Glu-(Thr, Ser_3, Glu_2, Val, $Isoleu_2$). Apparent ionization constants of this phosphopeptide have been determined potentiometrically. Probable linkages of the phosphate groups of the phosphopeptide have been discussed.

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Note added in proof. Since this manuscript was completed, Peterson, Nauman and McMeekin (J. Am. Chem. Soc. *80*, 95 (1958)) have reported the separation and amino acid composition of a pure phosphopeptide prepared from β-casein by the action of trypsin.

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Mitgeteilt am 4. Juni 1958 durch Hans v. Euler

Neue Dimethyl-chinolin-Derivate

Von Hans Hasselquist

Zu Untersuchungen verschiedener Arten von N-Oxyden hinsichtlich ihrer chemischen Eigenschaften und Reaktionen und ihrer anti-cancerösen Wirksamkeit wurde, wie bereits mitgeteilt, das früher nicht bekannte 7,8-dimethyl-5-nitrochinolin und dessen N-Oxyd (1) sowie das 5,6- Dimethyl-chinoxalin mit dessen Mono-N-oxyd (2) ferner das *p*-Phenanthrolin-di-N-oxyd (3) dargestellt. Bezüglich der Synthesen des sehr interessanten N-Oxyd-Lost (4) und der substituierten Chinolin-N-oxyde (5) sei auf die Arbeiten von M. Bergmann und von E. Ochiai verwiesen.

Zur Erweiterung der Kenntnisse über die im Benzolkern Dimethyl-Nitro-substituierte Chinoline und deren N-Oxyde, deren Methylgruppen zu Carboxyle oxydiert werden können (dieselben werden in dieser Weise als Alkalisalze leichter löslich, während die Chinolin-Derivate sonst schwerlöslich sind), wird hier die Darstellung der früher unbekannten Verbindungen 5,8-Dimethyl-7-nitro (III a) bzw. -7-Amino-chinolin und ihrer Acetyl-Derivate beschrieben.

Fukuoka (6) hat gefunden, dass das anti-cancerös 4-Nitro-chinolin-N-oxyd seine Eigenschaften beibehält und weniger toxisch und leichter löslich wird, wenn man Carboxyl in das 4-Nitro-chinolin-carbonsäure-N-oxyd einführt.

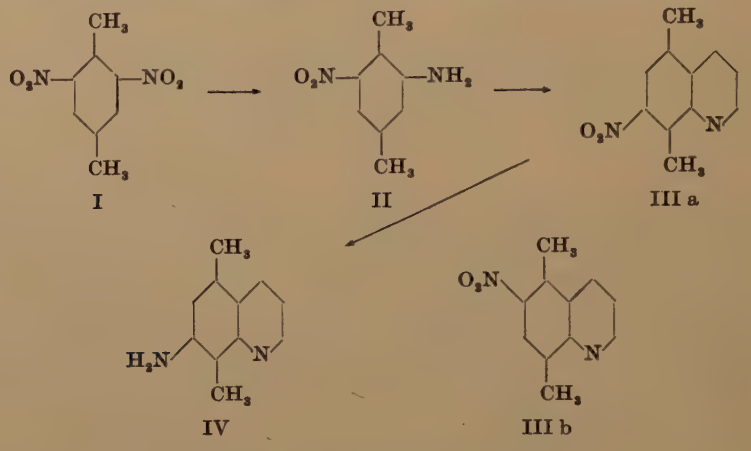
Unter dem in Benzolkern substituierten Chinolinen mit Dimethyl-nitro-Substitution sind nur 4 von den 12 möglichen Isomeren bekannt, nämlich

	Fp.			Fp.
5,6-Dimethyl-8-nitro-chinolin (7)	163°	resp.	8-amino-	—
5,8-Dimethyl-6-nitro-chinolin III b (8)	118°		6-amino-	175°C
7,8-Dimethyl-5-nitro-chinolin (3)	119°		5-amino-	140°C
7,8-Dimethyl-6-nitro-chinolin (8)	137°		6-amino-	163°C

Um das Auftreten von Isomeren der Darstellung von 5,8-Dimethyl-7-nitrochinolin zu vermeiden — es wäre bei der direkten Nitrierung zu erwarten — wurde zuerst (siehe nachfolgendes Schema) 1,4-Dimethyl-2,6-dinitro-benzol (I) im wesentlichen nach Fittig u. Mitarb. (9) dargestellt. Vgl. auch P. Jannasch (10). Die partielle Reduktion desselben zu 2-Amino-1,4-dimethyl-6-nitro-benzol (II) nach früheren Vorschriften unter Einleiten von Schwefelwasserstoff in die ammoniakalische Alkohol-Lösung (Fittig u. Mitarb. (9) und auch nach Kostanecki (11) gab sehr unbefriedigende Resultate mit sehr variierenden Ausbeuten.

Es zeigte sich, dass man die gesuchte Substanz leicht und schnell *in quantitativer Ausbeute* erhalten konnte, wenn man Dinitro-xylol mit siedender Lösung von Dinatriumsulfid in alkoholisch-wässriger Lösung reduzierte. Vergl. Brand (10), der *m*-Dinitro-benzol mit einer wässrigen Lösung von Natriumsulfhydrat zu *m*-Amino-nitro-benzol reduzierte. Bei der Skraupschen Synthese wird hier Nitro-

benzol — statt wie bei früheren Chinolin-Synthesen As_2O_5 — angewendet, da letztere Methode nur etwa 1% Ausbeute an 5,8-Dimethyl-7-nitro-chinolin (III a) ergab. Die Reduktion von III a mit $SnCl_2$ zu 5,8-Dimethyl-7-amino-chinolin (IV) konnte leicht mit fast quantitativer Ausbeute durchgeführt werden.



Beschreibung der Versuche

Darstellung von 1,4-Dimethyl-2,6-Dinitrobenzol (I)

11 ml *p*-Xylol wurden mit 30 mm konz. H_2SO_4 ($d = 1{,}84$), hierauf tropfenweise unter kräftigem Umrühren im Lauf von 3 Stunden mit 9 ml konz. HNO_3 ($d = 1{,}42$) versetzt; Temperatur wurde unter 20° gehalten durch Abkühlen eines äusseren Gefässes. Nach 3 Stunden wurde das ganze 30 Min. auf dem sidenden Wasserbad erwärmt, worauf alles mit dem 3fachen Vol. Wasser unter ausreichender Kühlung verdünnt wurde. Hierbei kristallisierte 1,4-Dimethyl-2,3- und 1,4-Dimethyl-2,6-dinitrobenzol in kleinen farblosen Kristallen aus, die abfiltriert, gut gewaschen und getrocknet wurden. Ausbeute an Nitroverbindungen 4 g.

Die Kristalle wurden 3- bis 4mal aus Äthanol umkristallisiert, wobei das schwerer lösliche 1,4-Dimethyl-2,6-dinitrobenzol in reiner Form erhalten werden konnte. Lange farblose stabähnliche Nadeln vom Schmpt. 126°C[1] Ausbeute an reinem 1,4-Dimethyl-2,6-dinitrobenzol 0,5 g.

Aus der Mutterlauge konnte 1,4-Dimethyl-2,3-dinitrobenzol in Form grosser farbloser Prismen vom Schmpt. 93° erhalten werden.

Teilweise Reduktion von 1,4-Dimethyl-2,6-dinitrobenzol zu 2-Amino-1,4-Dimethyl-6-nitro-benzol (II)

2 g 1,4-Dimethyl-2,6-dinitrobenzol (I) wurden in 40 ml siedendem Äthanol gelöst und tropfenweise im Laufe von 15 Min. mit einer Lösung von 7,2 g $Na_2S \cdot 9H_2O$ in 40 ml Wasser versetzt. Das ganze liess man noch weitere 30 Min.

[1] FITTIG, AHRENS und MATTHEIDES (9) geben folgende Schmelzpunkte an: für 1,4-Dimethyl-2,6-dinitrobenzol 123,5° und für 1,4-Dimethyl-2,3-dinitrobenzol 93°.

kochen und verdünnte die noch warme Lösung mit dem gleichen Volumen Wasser. Beim Abkühlen kristallisierte das Amino-nitro-xylol in Form langer gold-gelber Nadeln aus, die abfiltriert, mit Wasser gewaschen und getrocknet wurden. Die erhaltene Amino-nitro-Verbindung war vollständig rein und zeigte den von FITTIG, AHRENS und MATTHEIDES angegebenen Schmpt. 96–97°. Ausbeute quantitativ.

Die Substanz ist schwerlöslich in Wasser, ziemlich löslich in Petroleumäther, leicht löslich in Methanol, Äthanol, Aceton, Benzol und Chloroform. Sie lässt sich am besten aus Äthanol-Wasserlösung umkristallisieren.

Darstellung von 5,8-Dimethyl-7-nitro-chinolin (III a)

1 g 2-Amino-1,4-Dimethyl-6-nitrobenzol (II) wurden in 1,8 g Glyzerin und 0,31 ml Nitrobenzol gelöst. Unter Wasserkühlung wurde tropfenweise 0,81 ml konz. H_2SO_4 ($d = 1{,}84$) zugesetzt; das ausgefallene Sulfat des Amins wurde so fein als möglich verteilt. Man liess nun das ganze auf dem Ölbad bei 140° 2 Stunden stehen, wobei eine dunkelbraune bis schwarze dickflüssige Masse erhalten wurde, die man nach Abkühlen mit 100 ml Wasser verdünnte und möglichst fein verteilte. Das ganze wurde alkalisiert durch tropfenweisen Zusatz von 10%iger Natronlauge bis pH ~ 12; hierauf wurde das ganze 3mal mit Benzol extrahiert.

Die gesammelten Benzolschichten wurden in üblicher Weise getrocknet, filtriert und im Vakuum zur Trockne verdampft, wobei eine gelbe Kristallmasse entstand, welche aus wenig kochendem Methanol umkristallisiert wurde; dabei erhielt man die Chinolinverbindung in Form von über 2 cm langen hell gelbbraunen Nadeln, die abfiltriert mit wenig Methanol gewaschen und getrocknet wurden. Schmpt. 129°. Durch weiteres Umkristallisieren aus Methanol wurde der Schmpt. nicht geändert. Ausbeute 150 mg.

Die Substanz ist schwer löslich in Wasser, ziemlich leicht löslich in warmem Methanol, ziemlich löslich in Petroleumäther und leicht löslich in kaltem Benzol und Chloroform.

Analyse.	Ber. für $C_{11}H_{10}O_2N_2$	C 65,33 %	H 4,99 %	N 13,86 %
	Gef.	66,14	5,44	13.97

Darstellung von 7-Amino-5,8-Dimethyl-chinolin (IV)

0,2 g 5,8-Dimethyl-7-nitrochinolin (III a) wurden mit 3 ml HCl (2 : 1) versetzt; das entstandene HCl-Salz wurde so fein als möglich verteilt. Hierauf setzt man 0,7 g $SnCl_2 \cdot 2H_2O$ zu und liess das ganze 10 Min. mit Rückflusskühler kochen. Nach Abkülen wurde mit 50 ml Wasser verdünnt und das ganze unter Abkühlung mit 10%iger Natronlauge alkalisert, bis die zuerst entstehende Fällung von Stannihydrat sich zu Stannat gelöst hatte. Die Lösung wurde 3mal mit Benzol extrahiert, die gesammelten Benzolextrakte in üblicher Weise getrocknet und im Vakuum zur Trockne verdampft. Die übrigbleibende hellgelbe Kristallmasse wurde aus wenig Methanol umkristallisiert, wobei das Aminochinolin in Form kleiner hellgelber Stäbchen erhalten wurde. Dieselben wurden noch einmal aus Methanol umkristallisiert. Ausbeute annähernd quantitativ; schmilzt unter Zersetzung bei 135°.

Die Substanz ist schwer löslich in Wasser, etwas löslich in Petroleum-äther, leicht löslich in siedendem Methanol, leicht löslich in Benzol und Chloroform.

Bei längerem Stehen an der Luft färben sich die Kristalle an der Oberfläche intensiv gelb.

Analyse. Ber. für $C_{11}H_{12}N_2$ C 76,71% H 7,02% N 16,27%
Gef. 76,41 7,28 16,54

Acetylierung von 7-Amino-5,8-Dimethyl-chinolin (IV)

150 mg 7-Amino-5,8-Dimethyl-chinolin wurden mit 0,5 ml Essigsäure-anhydrid und einer Spur konz. Schwefelsäure auf dem siedenden Wasserbad 10 Min. erwärmt, wobei das Ausgangsmaterial in Lösung ging. Nach Abkühlen verdünnte man vorsichtig mit wenig Wasser, wobei die Acetylverbindungen in Form kleiner farbloser Nadeln ausfielen, die abfiltriert, mit wenig Wasser gewaschen und getrocknet wurden. Ausbeute an Rohprodukt 80 mg vom Schmpt. 199°. Die Substanz wurde aus wenig Benzol umkristallisiert.

7-Acetamino-5,8-Dimethyl-chinolin ist schwer löslich in Wasser, ziemlich löslich in Benzol und Methanol.

Analyse. Ber. für $C_{13}H_{14}ON_2$ N 13,08%
Gef. 13,19

Ich danke herzlichst Herrn O. HEIDENBERGER für unermüdliche Hilfe bei der Ausführung der Versuche.

Stockholm. Universität, Institut für organ.-chemische Forschung.

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On the bromination of laevulic acid

By Christoffer Rappe

With 13 figures in the text

As a starting material for further work, the present author needed 3-bromolaevulic acid. It was possible to prepare this acid by a method of Wolff (1) modified by Overend, Turton and Wiggins (2), Hurd and Ferraro (3) and Hellström (4). Laevulic acid was treated with bromine in cooled concentrated hydrochloric acid, the precipitated 3:5-dibromolaevulic acid was filtered off, and the acid filtrate was extracted with ether. The ether was evaporated and the residue was recrystallized from carbon disulfide. Hughes and Watson (5) made a kinetic investigation of the bromination.

The present author has made a series of brominations in hydrochloric acid under various conditions. The amount of precipitated 3:5-dibromolaevulic acid varied with the bromination temperature as stated by Overend *et al.* (2). In all experiments the yield of extraction residue after one extraction with ether was less than 50 %, and together with the 3:5-dibromolaevulic acid not more than about 60 % of the added bromine could be accounted for.

When the experiment was carried out in darkness or when hydroquinone was added, the yield of 3:5-dibromolaevulic acid was greater, while addition of benzoyl peroxide did not influence the yield. This perhaps depends on the small solubility of benzoyl peroxide in the reaction mixture. However, a radical-free medium does seem to favour the formation of 3:5-dibromolaevulic acid.

Repeated recrystallizations of the extraction residue gave no pure 3-bromolaevulic acid. By distillation, cf. Overend *et al.* (2) and Hellström (4), the purification also failed, because of decomposition.

The amount of ether used for the extraction was discussed by Hellström (4). He found that a second and third extraction improved the yield of the extraction residue, but lowered the yield after distillation somewhat. These results indicated that some other products than 3-bromolaevulic and 3:5-dibromolaevulic acid were formed. Therefore the acid solution already once extracted was continuously extracted with ether to identify these products, and after the ether was evaporated, an oily residue was obtained, which was called the exhaustive residue. Analyses indicated that this contained both bromine and chlorine and about 20 % of the added bromine was exchanged for chlorine. Separation of the chlorolaevulic acid from the bromolaevulic acid could not be accomplished either by recrystallization from carbon disulfide or by distillation. Including the halogen content of the exhaustive extraction residue increased the amount of accounted bromine to about 85 %.

This exhaustive extraction residue was studied together with laevulic, 3-bromo- and 3:5-dibromolaevulic acid by means of a new chromatographic method. When a pH-indicator was used as a spraying reagent, the exhaustive extraction residue gave

two spots, one of which had the same R_F value as laevulic acid, and the other was the chlorinated acid. The 3-bromolaevulic acid in the exhaustive extraction residue could be detected when a specific reagent for bromo-substituted acids, cf. Hashmi and Cullis (6), was used.

When this mixture, consisting of chlorinated acid (70 %), brominated acid (10 %) and laevulic acid (20 %), was treated with thiourea, cf. Conrad and Schmidt (7), 2-amino-4-methyl-5-carboxymethylthiazole was obtained in almost as good yield as from pure 3-bromolaevulic acid. The infrared spectra and mixed melting point showed that the two products were identical. Since this substance only can be obtained from 3-halogenated laevulic acids, the chloro-containing acid must be 3-chlorolaevulic acid.

To study this halogen exchange, 3-bromolaevulic acid was dissolved in concentrated hydrochloric acid and kept for one week then continuously extracted and worked up as usual. Potentiometric analyses showed that 70 % of the bromine was exchanged for chlorine during this treatment.

A similar exchange was studied by Thomson (8). He prepared 6-bromo-2:3-dichlorojuglone in good yield from 2:3:6-tribromojuglone by treating with hydrochloric acid. The two bromine atoms in α-positions to the carbonyl group are exchanged for chlorine. This exchange of bromine for chlorine is the reverse step of the Finkelstein reaction, and this reversible reaction was studied by Hannerz (9) on α-iodopropionic acid and potassium bromide.

This halogen exchange could be prevented when hydrobromic acid was chosen as reaction medium instead of hydrochloric acid. The present author found, however, that although the product from the bromination in hydrobromic acid after repeated crystallizations gave good analysis figures, the melting point showed a large range. This indicates that the product formed is a mixture of isomers. Chromatographic

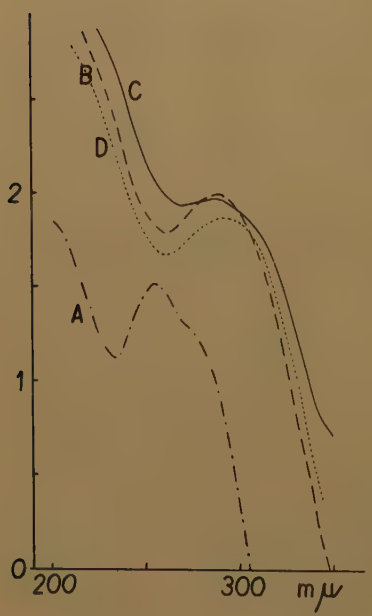


Fig. 1. UV-spectra of (*A*) laevulic acid, (*B*) 3-bromolaevulic acid, (*C*) 3:5-dibromolaevulic acid and (*D*) 5-bromolaevulic acid.

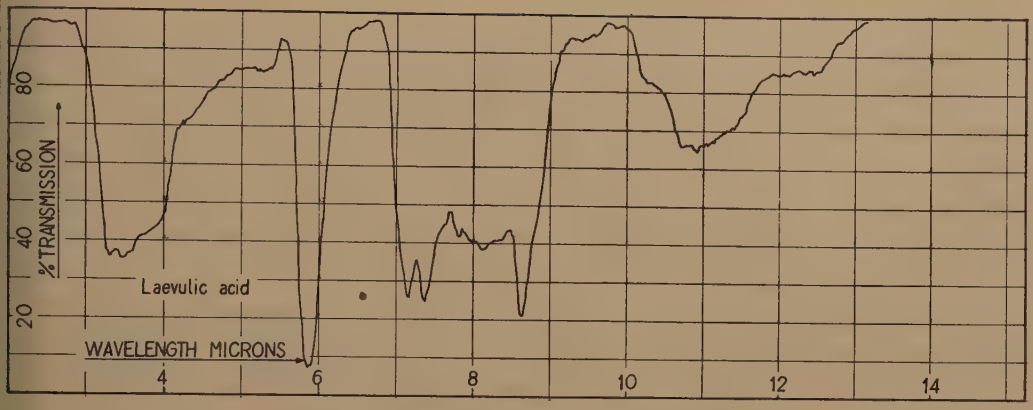


Fig. 2. IR-spectrum of laevulic acid in $CHCl_3$.

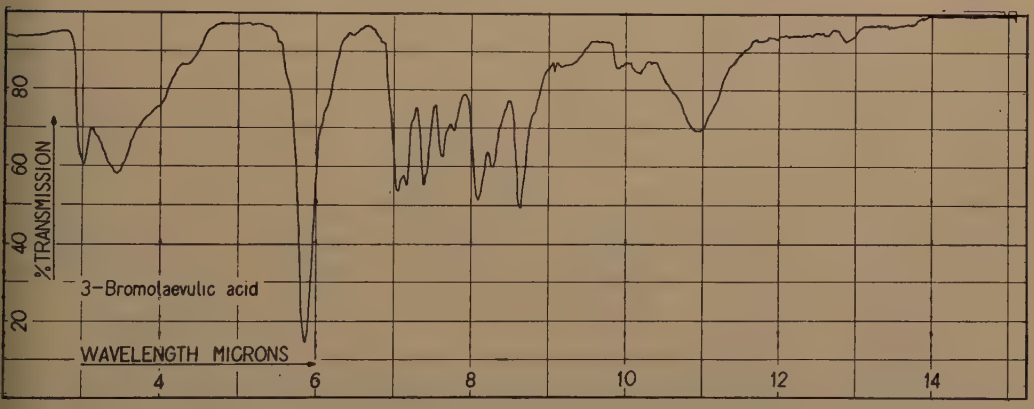


Fig. 3. IR-spectrum of 3-bromolaevulic acid in KBr.

analysis gave two spots, the greater one had the same R_F value as the 3-bromolaevulic acid from the bromination in hydrochloric acid.

Pure 3-bromolaevulic acid could be obtained in rather good yield when the bromination was done in 48 % hydrobromic acid and the product was purified in a modified way, see experimental part. From the bromination in 2 % hydrobromic acid, the other isomer could be obtained pure but in poor yield. It had the m.p. 78–79°C.

The ultraviolet spectrum of this latter bromolaevulic acid ($\lambda_{max} = 290$ mμ, log $\varepsilon = 1.9$) (curve D, Fig. 1) was studied together with those of laevulic acid (curve A; $\lambda_{max} = 256$ mμ, log $\varepsilon = 1.5$), 3-bromolaevulic acid (curve B; $\lambda_{max} = 288$ mμ, log $\varepsilon = 2.0$) and 3:5-dibromolaevulic acid (curve C; $\lambda_{max} = 288$ mμ, log $\varepsilon = 2.0$). The bathochromic shift was about the same for all the acids, and this indicates that the studied isomer is 5-bromolaevulic acid. 2-Bromolaevulic acid, which has the bromine atom in β-position to the carbonyl group, would probably not give as large a bathochromic shift as the acids with the bromine atom in α-position.

Jones and Cole (10) have found that the CH_3CO-group in infrared spectra has a strong representative top at 7.36–7.37 μ (1356–1359 cm^{-1}). In laevulic acid (Fig. 2)

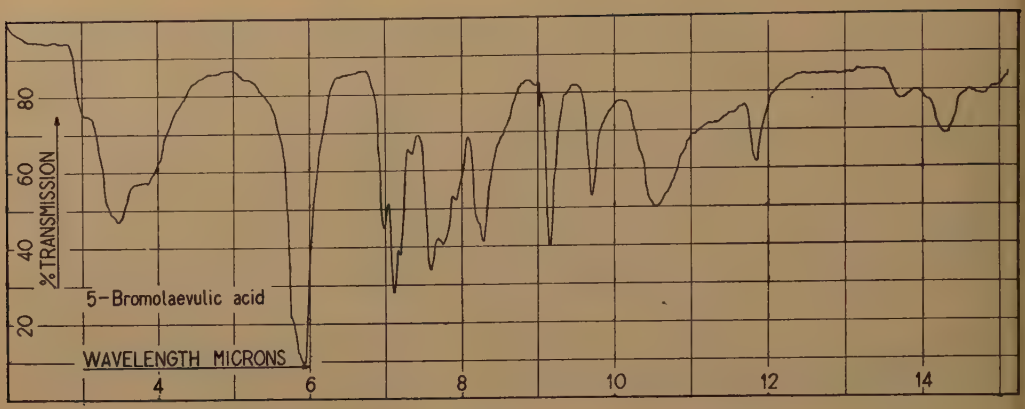


Fig. 4. IR-spectrum of 5-bromolaevulic acid in KBr..

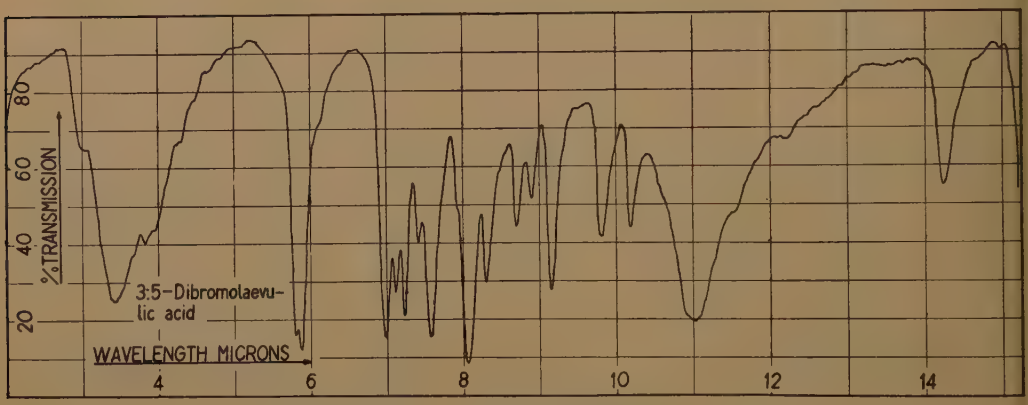


Fig. 5. IR-spectrum of 3:5-dibromolaevulic acid in KBr.

this top is at 7.37 μ (1358 cm^{-1}), 3-bromolaevulic acid (Fig. 3) has a top at 7.37 μ (1358 cm^{-1}) too, but the isomer studied (Fig. 4) and 3:5-dibromolaevulic acid (Fig. 5) only have weak bands at 7.34 μ (1362 cm^{-1}) and 7.42 μ (1350 cm^{-1}) respectively. This indicates also that the acid is 5-bromolaevulic acid.

The best evidence of the structure for 5-bromolaevulic acid could be obtained from its proton magnetic resonance (NMR-) spectrum (Fig. 6 and 7). For comparison the spectra of laevulic, 3-bromo- and 3:5-dibromolaevulic acid were also studied.

Laevulic acid (Fig. 8) has three peaks and their signal ratio is 1:4:3. Peak I can be assigned to the COOH-group, peak II to the two CH_2-groups and peak III to the CH_3-group. The CH_3-peak is a singlet because the adjacent carbon atom has no hydrogen atoms. The two CH_2-groups overlap each other because the two groups have a similar molecular surrounding. 3-Bromolaevulic acid (Fig. 9) has four peaks with the signal ratio 1:1:2:3, and the peaks can be assigned to (I) COOH-, (II) CHBr-, (III) CH_2- and (IV) CH_3-groups. 3:5-Dibromolaevulic acid (Fig. 10) has four bands too, and their signal ratio is 1:1:2:2. The bands can be assigned to (I) COOH-,

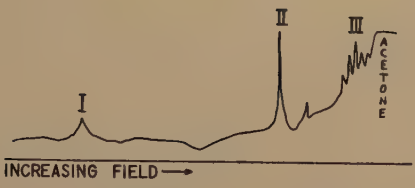


Fig. 6. NMR-spectrum of 5-bromolaevulic acid in *acetone*.

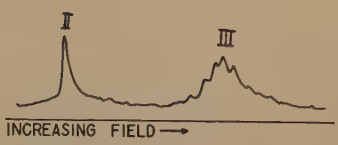


Fig. 7. NMR-spectrum of 5-bromolaevulic acid in *hydrobromic acid*.

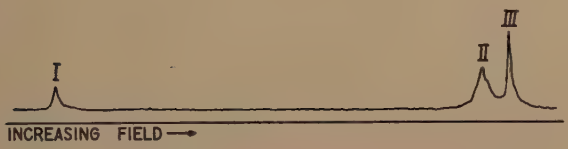


Fig. 8. NMR-spectrum of laevulic acid.

Fig. 9. NMR-spectrum of 3-bromolaevulic acid.

(II) CHBr-, (III) CH_2Br- and (IV) CH_2-groups. This spectrum proves that the bromine atoms are located on different sides of the keto-group, i.e. the structure is not 3:3- or 5:5-dibromolaevulic acid, but this spectrum does not exclude the 2:5-isomer. This isomer, however, is not probable, because ketones are brominated in the α-positions to the carbonyl group, and furthermore the di-acid can be synthesised from bromination of 3-bromolaevulic acid (11, 5). This experiment has been confirmed by the present author.

It was impossible to find a solvent for 5-bromolaevulic acid, which had no disturbing groups. The spectrum had to be recorded with both acetone (Fig. 6) and hydrobromic acid (Fig. 7) as solvent. The signal ratio I:II:III was found to be 1:2:4, and the peaks can be assigned to COOH-, CH_2Br- and the two CH_2-groups respectively. As in laevulic acid, the two CH_2-groups overlap each other, and the CH_2Br-peak is a singlet, compare peak IV Fig. 9 and peak III Fig. 10.

2-Bromolaevulic acid would be expected to give a spectrum like that of 3-bromolaevulic acid, containing four peaks with the signal ratio 1:1:2:3, and the singlet peak furthest to the right.

The present author made three brominations in varying dilution of hydrobromic acid. The yields of 3:5-dibromolaevulic acid were determined by weighing and those of 5-bromolaevulic acid by infrared analyses of the crude products. These results are collected in Table 1.

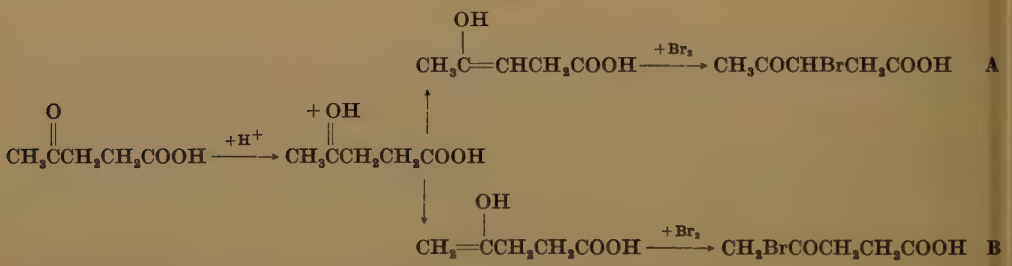
Fig. 10. NMR-spectrum of 3:5-dibromolaevulic acid.

Table 1

% HBr	$CH_3BrCOCHBrCH_2COOH$	$CH_3BrCOCH_2CH_2COOH$
66	28.3 g	3 % 0.9 g
48	12.0 g	7 % 4.1 g
2	0 g	24 % 17.7 g

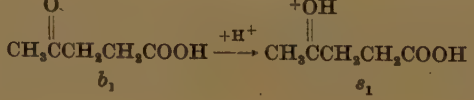
Overend *et al.* (2) stated that the amount of 3:5-dibromolaevulic acid formed in hydrochloric acid varied with the bromination temperature. In Table 1 it is shown that in hydrobromic acid this amount also depends on the dilution of the hydrobromic acid. The amount of 5-bromolaevulic acid is found to be dependent on the dilution too.

The formation of the two isomers is in agreement with the accepted theory of acid catalyzed bromination of ketones (12, 13, 14). It is known that when a ketone is brominated in acid media, the bromine reacts with the enolic form of the ketone. In the case of laevulic acid the enolisation can follow two routes, resulting in 3-bromolaevulic acid (A) and 5-bromolaevulic acid (B).



Because of hyperconjugation the enol favoured in the bromination is the one which has the double bond between the carbonyl carbon and the most substituted α-carbon atom, cf. Cardwell and Kilner (15); in the case of laevulic acid, route A. In contrast to acetoacetic acid, the enolisation of laevulic acid is not influenced by the carboxyl group, because of the two screening CH_2-groups. Butanone-2 gives the ratio 1-brominated:3-brominated ketone = 27:73 (16), and the ratio of the two bromolaevulic acid isomers is found to be about the same when the bromination is performed in dilute hydrobromic acid.

The increasing amount of 3:5-dibromolaevulic acid with the increasing strength of the hydrobromic acid is more difficult to explain. Dewar (12) states, that when a ketone is brominated in *acid* solution, the monobromo ketone can be isolated, since this reacts more slowly with the bromine than the unbrominated ketone does. The explanation of this is that the bromination probably takes part through a cation, and the cation corresponding to the bromo ketone is a stronger acid than the one corresponding to the ketone (s_2 is stronger than s_1), and usually the latter is formed.



$$\underset{b_2}{CH_3\overset{\overset{O}{\|}}{C}CHBrCH_2COOH} \xrightarrow{+H^+} \underset{s_2}{CH_3\overset{\overset{+OH}{\|}}{C}CHBrCH_2COOH}$$

In agreement with this, laevulic acid in 2 % hydrobromic acid gives s_1. The higher acidity in 66 % hydrobromic acid forces the weaker base b_2 to accept protons too, and hence the quotient s_2/s_1 increases. The discussion is the same when 5-bromolaevulic acid is brominated.

When Wolff (1) treated acetylacrylic acid with concentrated hydrobromic acid, he could isolate a bromolaevulic acid, which he supposed to be 2-bromolaevulic acid, but he had no constitutional proof. Wolff (1) quoted the melting point of this acid as 79–80°C, that is, the same as that of the 5-bromolaevulic acid mentioned above. Mixed melting point and infrared spectra showed that the acid obtained according to Wolff (1) and the 5-bromolaevulic acid were identical.

Instead of adding hydrogen bromide over the double bond, giving 2- or 3-bromolaevulic acid as Wolff (1) supposed, acetylacrylic acid seems to react in its enolic form under 1.4-addition

$$CH_3COCH{=}CHCOOH \overset{H+}{\rightleftarrows} CH_2{=}\underset{\underset{OH}{|}}{C}CH{=}CHCOOH \xrightarrow{+HBr} CH_2BrC{=}\underset{\underset{OH}{|}}{C}HCH_2COOH \rightleftarrows$$

$$\rightleftarrows CH_2BrCOCH_2CH_2COOH$$

The enol formation of acetylacrylic acid is favoured by the strong acid media, in which the addition takes part.

Discussion of the infrared spectra

Bellamy and coworkers (17, 18) have studied the influence of α-halogen atoms on the carbonyl frequencies in the infrared. In α-halogenated ketones, in which a negatively charged halogen atom is near the carbonyl-oxygen atom, the induced charges would diminish the carbonyl bond polarity and hence the frequency would rise. Bellamy *et al.* (17, 18) have found this to be the case when the carbonyl-oxygen and

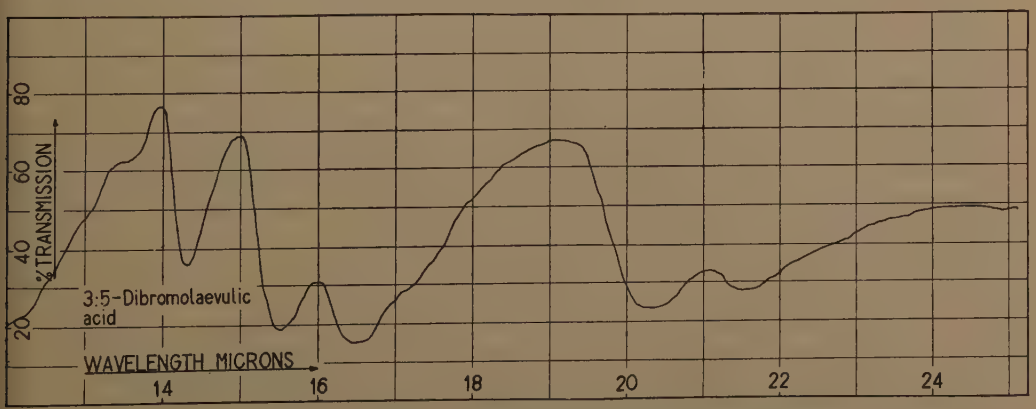


Fig. 11. IR-spectrum of 3:5-dibromolaevulic acid in KBr.

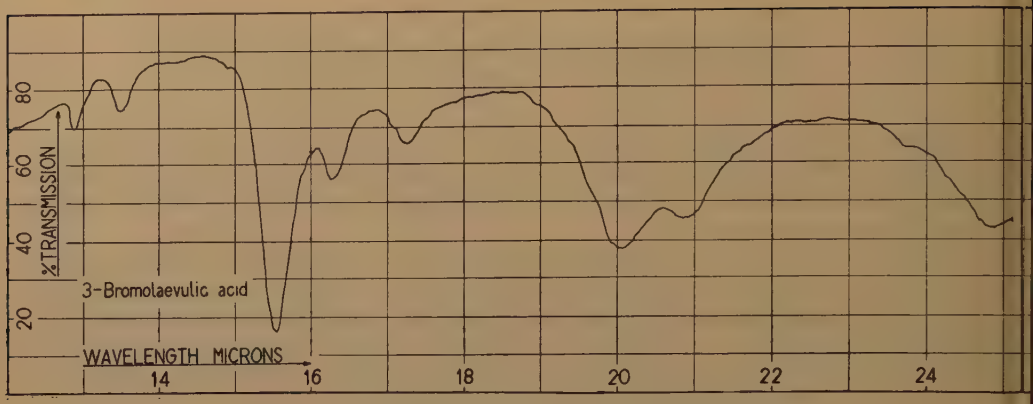


Fig. 12. IR-spectrum of 3-bromolaevulic acid in KBr.

the halogen atom are in a *s-cis*-configuration, but when they are in a *gauche* configuration the carbonyl frequency is unchanged. Laevulic acid (Fig. 2) has its carbonyl top at 5.85 μ (1710 cm^{-1}) and 3-bromolaevulic acid (Fig. 3) has an unchanged carbonyl frequency at 5.85 μ (1710 cm^{-1}) and this indicates a *gauche* configuration for this acid. The same is valid for 5-bromolaevulic acid (Fig. 4) where the carbonyl top is at 5.90 μ (1695 cm^{-1}). 3:5-Dibromolaevulic acid (Fig. 5) has two bands in this region, one at 5.79 μ (1727 cm^{-1}) and another at 5.88 μ (1700 cm^{-1}). The former can be ascribed to a raised carbonyl frequency and the latter to a carboxyl band.

The structure of 3:5-dibromolaevulic acid could be confirmed from its infrared spectrum in the potassium bromide region. Bellamy (19) has stated that the presence of more than one bromine atom at the same carbon atom causes frequency shifts of the C-Br absorption. The C-Br absorptions of 3:5-dibromolaevulic acid (Fig. 11) are 15.52 μ (644 cm^{-1}) and 16.44 μ (608 cm^{-1}) and these agree with those of 3-bromolaevulic acid (Fig. 12): 15.53 μ (643 cm^{-1}), 16.29 μ (614 cm^{-1}) and 17.26 μ (579 cm^{-1}).

Experimental part

The micro analyses were carried out by the Analytical Laboratory at the Chemical Institute, University of Uppsala, the argentiometric titrations by the present author. The melting point measurements were performed by means of the Kofler microscopic method. The infrared spectra were obtained with a Perkin Elmer model 21 spectrophotometer provided with a sodium chloride or a potassium bromide prism, and the ultraviolet spectra with a Beckman model DU spectrograph with readings generally every 5 mμ. The proton magnetic resonance spectra were determined with a Varian model V-4300B high-resolution spectrometer fitted with a field-sensing stabilizer, operating at 40 Mc/s.

Bromination of laevulic acid in hydrochloric acid

To a solution of laevulic acid (50 g) in 140 ml of concentrated hydrochloric acid, 22.5 ml of bromine was added dropwise. The reaction vessel was cooled with an ice-salt bath. After the addition was finished, the reaction mixture was inocculated

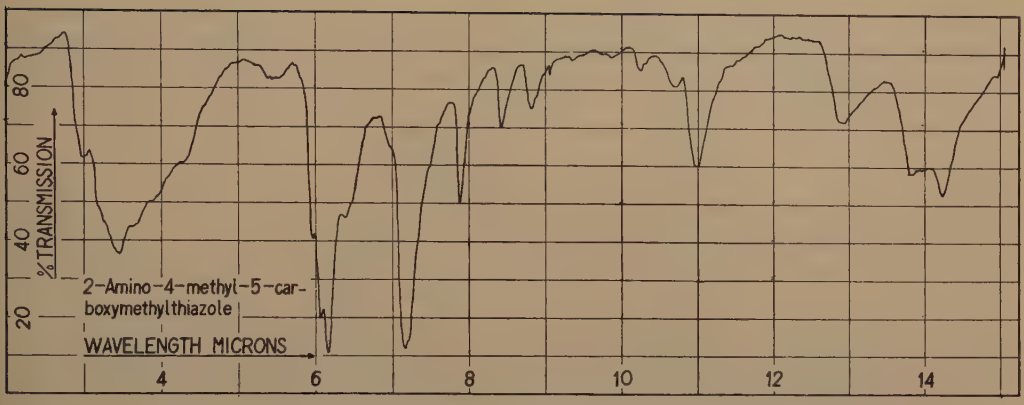


Fig. 13. IR-spectrum of 2-amino-4-methyl-5-carboxymethylthiazole in KBr.

with 3:5-dibromolaevulic acid and left to crystallize for 24 hours at 0°C. The precipitated 3:5-dibromolaevulic acid was filtered off, dried in vacuo over potassium hydroxide, and weighed. Yield: 7.0 g. After 500 ml of water had been added, the filtrate was extracted with 150 ml of ether. The ether extract was dried with magnesium sulphate, and the ether was removed under vacuum until a constant refractive index ($n_D^{25} = 1.495$) was reached. The residue weighed 44.0 g and was recrystallized from carbon disulfide (10 ml/1 g). After seven repeated recrystallizations 11.8 g m.p. 46–52°C remained.

Anal. $C_5H_7O_3Br$ (195.0) Calc. C 30.77; H 3.62; Br 40.98
Found C 29.88; H 3.55; Br 42.20

Wolff (1) quoted the m.p. of 3-bromolaevulic acid as 59°C. Overend *et al.* (2) quoted 53° and Hurd *et al.* (3) quoted 55–56°C. The m.p. and the analyses showed that the acid was not pure.

The reaction mixture, once extracted with ether (about 700 ml), was continuously extracted with ether for 15 hours. The ether was removed under vacuum until constant refractive index ($n_D^{25} = 1.460$). This residue was an oily liquid and weighed 26.8 g. It was distilled in a modified Claisen flask and no decomposition was observed. After a fore-run of 5.7 g, a fraction of 12.5 g b.p. 127–130°C at 2.2 mm ($n_D^{25} = 1.463$) was collected.

Anal. $C_5H_7O_3Br$ (195.0) Calc. C 30.77; H 3.62; Br 40.98
$C_5H_7O_3Cl$ (150.5) Calc. C 39.87; H 4.69; Cl 23.56
$C_5H_8O_3$ (116.1) Calc. C 51.70; H 6.94
Found C 41.47; H 5.04; Br 3.57; Cl 16.68

These figures indicate that the distilled residue consists of about 10 % 3-bromolaevulic acid, 70 % 3-chlorolaevulic acid and 20 % laevulic acid.

Bromination of laevulic acid in hydrobromic acid

A. The synthesis was performed in the same way as described above for bromination in hydrochloric acid. 100 ml of hydrobromic acid (48 %) was used, and 12.0 g of

dried 3:5-dibromolaevulic acid was collected. Extraction with ether (150 + 4 × 100 ml) gave 59 g of a residue, which after two crystallizations from carbon disulfide gave 52.1 g m.p. 32–50°C. After three additional crystallizations, 43.5 g acid with a m.p. 35–49°C was collected.

Anal. $C_5H_7O_3Br$ (195.0) Calc. C 30.77; H 3.62; Br 40.98
Found C 30.84; H 3.56; Br 41.15

Infrared analyses showed that the crude residue contained 7 % 5-bromolaevulic acid.

B. Laevulic acid (50 g) was dissolved in 100 ml of hydrobromic acid (66 %) and cooled (−5°C). 22.5 ml of bromine was added, and when the addition was ended, 500 ml of water was added. Upon cooling 3:5-dibromolaevulic acid precipitated and was filtered off, dried and weighed, 28.0 g. The solution was extracted with ether (150 + 4 × 100 ml) and the crystallized residue (41 g) was shaken for 2 hours with carbon tetrachloride (3 ml/1 g). The undissolved 3:5-dibromolaevulic acid (0.3 g) was filtered off and 400 ml of petrol (b.p. 60–85°C) was added to the filtrate. An oil separated, which crystallized in a freezing box (34 g). Infrared analysis: 3 % 5-bromolaevulic acid.

C. The bromination was performed in 660 ml of water and 20 ml of hydrobromic acid (48 %) at room temperature. All the bromine (22.5 ml) was added at once and after five days the bromine colour had disappeared. No 3:5-dibromolaevulic acid could be obtained. The ether extraction residue weighed 74 g and contained 24 % 5-bromolaevulic acid.

For the analyses of the mixtures the base-line method (20, 21) was accomplished. For determination of the 5-isomer, the top at 9.16 μ (1092 cm^{-1}) was used. A calibration curve was constructed from synthetic mixtures of 3- and 5-bromolaevulic acid in carbon tetrachloride.

Purification of the isomer-mixtures

A. *3-Bromolaevulic acid*

The analyzed isomer-mixtures could be purified by shaking with carbon disulfide (20 ml/g) at room temperature. Crystals could be filtered off after the carbon disulfide solution had been left for crystallization at 0°C for 24 hours. After two purifications in this way the crystals had m.p. 48–52°C. When petrol (b.p. 60–85°C) was added to the carbon disulfide and when the solution was cooled at −20°C, more crystals could be harvested, which could be purified in the same way. The amounts of 3-bromolaevulic acid of m.p. 48–52 from the different syntheses were (A) 32 g, (B) 12 g and (C) 16 g. Repeated crystallizations from carbon disulfide raised the m.p. to 52–54°C.

B. *5-Bromolaevulic acid*

This acid could be obtained from the bromination in 2 % hydrobromic acid. When this crude product was shaken with two smaller portions of carbon disulfide (10 ml/g) crystals could be obtained by cooling the second portion, which after three recrystallizations from petrol (b.p. 60–85°C) (100 ml/1 g) had the m.p. 78–79°C. From 10 g of residue, 0.3 g of pure 5-bromolaevulic acid could be obtained.

Anal. $C_5H_7O_3Br$ (195.0) Calc. C 30.77; H 3.62; Br 40.98
Found C 31.22; H 3.69; Br 40.78

From the first portion of carbon disulfide 3-bromolaevulic acid could be obtained. Small amounts of the other isomer depressed the melting point. Melting ranges of mixtures of 3-and 5-bromolaevulic acid:

% 3-Bromolaevulic acid	Melting range °C
96	41–54
91	33–51
10	40–76
5	44–78

Treatment of 3-bromolaevulic acid with hydrochloric acid.—9.0 g of 3-bromolaevulic acid was dissolved in 50 ml of concentrated hydrochloric acid and kept for one week at room temperature. Then 50 ml of water was added and the solution was continuously extracted with ether for 48 hours. The ether was removed under vacuum until constant refractive index ($n_D^{25} = 1.475$). The residue weighed 7.0 g. Titrated potentiometrically: 14.7 % Br and 15.8 % Cl. Amount recovered halogenolaevulic acid: 93 %.

3:5-Dibromolaevulic acid.—The dried 3:5-dibromolaevulic acid was recrystallized from water: 25 g of acid in 100 ml of water gave about 19 g of product. After three recrystallizations a m.p. 111–113°C was obtained. Wolff (22) quoted 114–115°C and Claeson (23) 113–114°C.

Paper chromatography

A method for separating halogenated organic acids has been suggested by Hashmi *et al.* (6). As a developing solvent they used *n*-propanol (70 %) and ammonia (30 %), but this method was not feasible in the present case because the ammonia reacted with the acids. Amino acids could be detected by spraying with ninhydrin, and ammonium salts by blind tests. However, a developing solvent prepared in the following way was found to be suitable: 60 ml of benzene mixed with 30 ml of *cyclo*-hexane was saturated with water and then mixed with 10 ml of ordinary acetic acid. Whatman's No. 1 filter paper and the descending method were used, and the samples, not exceeding 50 μg, were applied as 1 % ether solutions.

A. *Investigation of the exhaustive extraction residue*

After drying, the chromatograms were sprayed with bromophenol blue in ethanol, 0.04 %, cf. Linskens (24). The following R_F values were found:

3:5-Dibromolaevulic acid	3-Bromolaevulic acid	Exhaustive extraction residue	Laevulic acid
0.62	0.54	0.44 and 0.23	0.21

When fluorescein was used as spraying reagent, cf. Hashmi *et al.* (6), and the papers were thereafter treated with chlorine, the following R_F values were found:

3:5-Dibromolaevulic acid	3-Bromolaevulic acid	Exhaustive extraction residue	Laevulic acid
0.62	0.54	0.55	—

The chromatograms had to be sprayed with ammonia before they were sprayed with fluorescein, or else no spots could be found. Hashmi *et al.* (6) suggested that the chromatograms should hang in the chlorine tank for about 5 seconds, but 1 second is enough, otherwise the spots disappear.

B. *Investigation of the crude product (48 % HBr)*

The chromatograms were made as described above. pH-indicator was used. The following R_F values were obtained:

3-Bromolaevulic acid	5-Bromolaevulic acid according to Wolff	Crude product (48 % HBr)
0.54	0.39	0.40 and 0.54

Preparation of 2-amino-4-methyl-5-carboxymethylthiazole

Following the directions given by Conrad *et al.* (7) for the synthesis of this compound from 3-bromolaevulic acid and thiourea, the following preparations were made.

A. 3-Bromolaevulic acid m.p. 48–52°C (4.0 g) was dissolved in water (10 ml) and poured into a solution of thiourea (1.6 g) in water (5 ml). After 24 hours at room temperature, the solution was neutralized to pH 5 with a sodium bicarbonate solution, and a faint yellow substance (2.9 g) crystallized. Recrystallization from water (150 ml) gave colourless crystals, with a temperature of decomposition varying with the heating rate. Slowly heated the crystals decomposed at 256–258°C, but in a preheated chamber (250°C) at 264–266°C. Conrad *et al.* (7) reported a m.p. 259–260°C. Yasuda (25) gave 274–275°C, in addition attributing this m.p. to Conrad *et al.* (6). Yield = 82 %. The adsorption in ultraviolet has λ_{max} at 268 mμ. The adsorption in infrared is shown in Fig. 13.

Anal. $C_6H_8N_2O_2S$ (172.1) Calc. C 41.83; H 4.69; N 16.28; S 18.62
Found C 41.48; H 4.74; N 16.19; S 18.24

B. The exhaustive extraction residue (3.3 g), thiourea (1.6 g), and water (15 ml) gave a thiazole compound; the crude product weighed 2.2 g. Two crystallizations from water gave a product, decomp. 256–259°C (slowly heated), 264–266°C (rapidly heated). Mixed with the product from A, decomp. 262–264°C. Yield 62 %.

Anal. $C_6H_8N_2O_2S$ (172.1) Calc. C 41.83; H 4.69; N 16.28; S 18.62
Found C 41.63; H 4.75; N 16.20; S 18.33

SUMMARY

When laevulic acid is brominated in hydrochloric acid, 3-chlorolaevulic acid is also formed. Better yields of 3-bromolaevulic acid are obtained when laevulic acid is brominated in hydrobromic acid. From this synthesis 5-bromolaevulic acid could also be isolated. The structure of this compound could be established by ultraviolet, infrared and proton magnetic resonance spectra. This acid was identical with the bromolaevulic acid obtained according to Wolff from acetylacrylic acid and hydrobromic acid, and by him tentatively identified as 2-bromolaevulic acid. Evidence has been found that acetylacrylic acid adds hydrogen bromide in the 1:4-positions through its enolic

form. The structure of 3:5-dibromolaevulic acid has been confirmed by spectroscopic methods. 2-Amino-4-methyl-5-carboxymethylthiazole is prepared from the 3-halogenated laevulic acids. A new chromatographic method for halogenated organic acids is described.

ACKNOWLEDGEMENTS

This investigation was suggested by Professor Nils Hellström. The present author is much indebted to him for valuable advices and interesting discussions. Thanks are also due to Professor Arne Fredga for his kind interest. The author is indebted to Dr. Ragnar Hoffman for taking the proton magnetic resonance spectra and for his expert advice in connection with them, and to Mr. Stig Bergvall for recording the infrared spectra and quantitative infrared analyses. The micro analyses were done by Dr. A. Bengtsson.

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Institute of Organic Chemistry, Royal Agricultural College, Uppsala, May 1958.

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N-Oxyde von Wirkstoffen des oxydo-reduktiven Stoffwechsels

Von Hans von Euler und Hans Hasselquist

Mit 3 Figuren im Text

Welche Einwirkungen auf den Stoffwechsel man bei der experimentellen Bearbeitung therapeutischer, genetischer oder anderer Probleme auch hervorrufen will, stets wird man versuchen, das Ziel durch Angriff auf *Enzym-Systeme* zu erreichen, wobei in erster Linie deren prosthetische Gruppen in Betracht kommen.

Das Verhalten von *N-Oxyden der Cozymase-Komponenten* und anderer Wirkstoffe lassen es möglich erscheinen, dass gewisse Phasen des oxydoreduktiven Stoffwechsels durch in vivo gebildete *N*-Oxyde vermittelt werden (H. v. Euler, Vortrag Lindau 1958). Die biologisch ausserordentlich wichtige Synthese der Cozymase verläuft, wie nunmehr bewiesen ist, im Zellkern, in dem sich dieses am meisten verbreitete unter den Coenzymen in verhältnismässig grossen Mengen (1) findet.

Während die rein chemische *Synthese der Cozymase* durch Sir Alexander Todd (2) in ausgezeichneter Weise durchgeführt ist, bleiben bezgl. der in vivo verlaufenden Synthese noch wesentliche Fragen offen (3). Betrachtet man das energiereiche Adenosintriphosphat als den einen der Ausgangsstoffe, so kann man als zweite Komponente der Synthese das Nikotinsäureamid-nukleotid (unter Bildung von Pyrophosphat) oder das Nikotinsäureamid-nukleosid annehmen. Letzteres Nukleosid ist von Fr. Schlenk (4) als Spaltprodukt der Cozymase isoliert und analysiert worden; es schliesst sich in jeder Hinsicht den Karrerschen Modell-Verbindungen (5) gut an.

Wie wir (6) in früheren Mitteilungen nachgewiesen haben, bilden Nikotinsäure und Nikotinsäureamid die gut definierten *N*-Oxyde $C_6H_5O_3N$ bezw. $C_6H_6O_2N_2$. Auch die Adenosin-Komponente der Cozymase liefert *N*-Oxyde, wie von uns (6) und etwa gleichzeitig von M. A. Stevens u. Mitarb. (7) gezeigt wurde.

Natürlich können auch andere Reaktionswege als die eben erwähnte für die in vivo verlaufende Synthese der Cozymase in Betracht kommen (8). Ob bei diesen die *N*-Oxyde der Komponenten eingreifen lässt sich noch nicht entscheiden (Euler, Vortrag in Lindau; Naturwiss. Rundschau, im Druck).

Was die Ausgangs-Substanzen der Cozymase-Synthese betrifft, so sei in Zusammenhang mit der Nikotinsäure und ihrem *N*-Oxyd auch die Hydroperoxyd-Wirkung auf die Pyridin-Aldehyde beschrieben, dessen Oxydation zur Nikotinsäure bzw. Isonikotinsäure führt.*

* Wir verdanken die beiden Pyridin-Aldehyde dem freundlichen Entgegenkommen der Chemischen Fabrik Dr. F. Raschig, Ludwigshafen/Rhein.

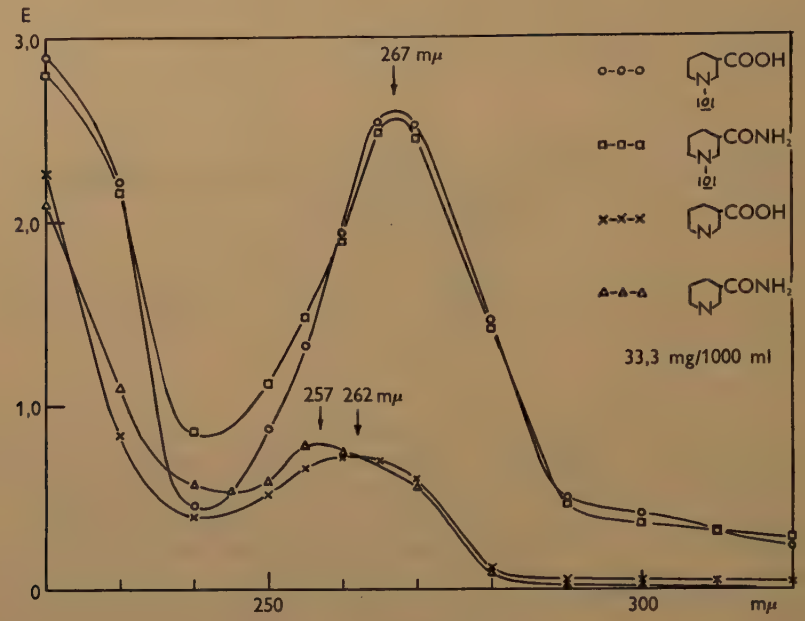

Fig. 1.

Pyridin-3-Aldehyd wurde, wie früher beschriebene Pyridin-Derivate und Hydroperoxyd in Eisessig behandelt und lieferte die Produkte: Nikotinsäure und Nikotinsäure-N-oxyd.

Pyridin-4-Aldehyd; Übergang in Isonikotinsäure (296). 1 ml Pyridin-4-Aldehyd in 5 ml Eisessig wurde mit 1 ml Hydroperoxyd-Lösung 12 Stunden auf 65° erwärmt. Die Pyridin-4-carbonsäure auskristallisierte (Schmp. 304°). Nach Zusatz von 1 ml Hydroperoxyd-Lösung, wurde die Reaktionsmischung nochmals 12 Stunden auf 65° erwärmt. Durch Eindunsten im Vakuum zur Trockne, wurde eine weisse Kristallmasse erhalten. Nach Zusatz von Eisessig wurden die Kristalle abfiltriert und gewaschen. Nach zweimaligen Umkristallisieren aus wenig Wasser erhielt man lange farblose Stäbchen vom Schmp. 271°.

Die Substanz 296 ist löslich in warm Methanol, doch schwer löslich in allen üblichen organischen Lösungsmitteln. Ausbeute kvantitativ.

Analyse Subst. 296:

Ber. für $C_6H_5O_3N$	C 51,80 %	H 3,62 %	N 10,01 %
Gefunden	51,51	4,05	9,87

Unter den am oxydo-reduktiven Stoffwechsel beteiligten Dinukleotiden ist an zweiter Stelle zu nennen das *Adenin-riboflavin-dinukleotid.*

Es fungiert, abgesehen von seiner Stellung im „alten gelben Ferment" als prosthetische Gruppe mehrerer Enzyme, darunter der Diaphorase, ferner der *D*-Aminosäure-Oxydase (aus der Abraham (9) die Adenosin-monophosphorsäure freimachte), der Cytochromreduktase und anderer.

Die Synthese des Riboflavin-adenin-dinukleotids verläuft nach Kornberg durch die Reaktion von Riboflavin-mononukleotid mit ATP.

Riboflavin wurde von uns mit dem gleichen Verfahren behandelt durch welches wir das Nikotinsäureamid in das *N*-Oxyd übergeführt haben:

0,8 g Riboflavin in 100 ml Eisessig suspendiert, liessen wir mit 20 ml einer 30%igen Hydroperoxydlösung bei 80° ca 15 Stunden stehen, wobei alles Riboflavin in Lösung ging.

Aus der im Vakuum bis auf 2 ml eingeengten Lösung fielen nach Zusatz von 3 ml Methanol kompakte Krystalle vom Schmpt. 258° aus. Umkrystallisation dieses Rohproduktes (0,4 g) aus Wasser ergab lange, feine Nadeln vom Schmpt. 278°; darauf folgendes Umkrystallisieren aus Pyridin erhöhte den Schmpt. weiter auf 284°.

Analyse: Berechnet für $C_{13}H_{18}O_{10}N_4$ C 40,00 % H 4,65 % N 14,36 %
Gef. 39,75 4,36 13,83

Eine Molekular-Gewichts-Bestimmung (Gefrierpts-Erniedrigung in Phenol) ergab:

Subst. 42,3 mg Phenol 2325,1 mg
$\Delta° = 0{,}37°$
M_{ber} 390 M_{gef} 360

Umsetzung von ATP *und* AMP *mit Hydroperoxyd*

20 mg ATP in 0,5 ml Eisessig + 0,1 ml einer 30%igen Hydroperoxyd-lösung reagierten 24 Stunden und dann die Lösung wurde zur Papierchromatographie verwendet. 20 mg AMP in der gleichen Weise behandelt (Munktells OB) A. Flüssigkeit: 1 Teil Wasser, 3 Tl. Ameisensäure und 16 Tl. Methanol nach R. S. Bandurski und B. Axelrod (J. Biol. Chem 193, 405 (1951).

Entwickler I: 5 ml 60%ige Perchlorsäure-Lös. + 10 ml 1-*n* HCl + 25 ml 4%ig Ammoniummolybdatlös. mit Wasser auf 100 ml verdünnt. Nach Trocknen des Chromatogrammes bei 85° während 7 Minuten wurde es während 5 Min. einer H_2S-Atmosphäre ausgesetzt. wobei das Phosphat und die Phosphorsäureester mit intensiv blauer Farbe hervortraten.

Entwickler II: Das Papier wird mit einer 0,25 m $Hg(NO_3)_2$-Lös. in 0,5 m HNO_3 bespritzt, dann gewaschen in 0,5 m HNO_3-Lösung hierauf mit Wasser und schliesslich in eine wässrige Ammoniumsulfidlösung eingetaucht, wobei die Purine als graue Flecken hervortreten (10).

Mit Flüssigkeit A und Entwickler I wurden Chromatogramme folgender Substanzen hergestellt:

	ATP	Blauer	Flecken	$R_f = 0{,}86$
H_2O_2 beh.	ATP	»	»	$R_f = 0{,}86$ Orthophosph., gelb dann blau R_f 0,90
	AMP	»	»	$R_f = 0{,}86$
H_2O_2 beh.	AMP	»	»	$R_f = 0{,}86$ Ortophosph.-Flecken, $R_f = 0{,}90$
Orthophosph.		»	»	
Adenin:		—	—	
Adenin-*N*-Oxyd:		—	—	
Adenosin:		—	—	

Das Chromatogramm mit Flüssigkeit A und Entwickler I zeigt:

1. ATP und AMP spalten in Eisessig mit Hydroperoxyd bei 20° PO_4^{3-} ab.
2. Die nach Abspaltung von Phosphat gebildeten Stoffe haben den gleichen R_f-Wert wie das unbehandelte ATP bzw. AMP; ein Unterschied mit der Flüssigkeit A trat also nicht hervor.
3. Adenin, Adenin-*N*-Oxyd und Adenosin gaben mit Entwickler I keinen Flecken.

Um die unter 3. erwähnten Substanzen entwickeln zu können wurde ein neuer Versuch ausgeführt mit der Flüssigkeit A und unter Verwendung von *Entwickler II*, durch den indessen PO_4^{3-} nicht erfasst wird. Letzterer Umstand spielt hier keine Rolle, da eine Scheidung von PO_4^{3-} im vorhergehenden Chromatogramm deutlich hervortrat.

Adenin $R_f = 0{,}86$.
Adenin-*N*-Oxyd $R_f = 0{,}89$.

Adenosin wegen zu kleiner Konzentration nicht feststellbar. Das Chromatogramm mit Flüssigkeit A und Entwickler II zeigen:

1. Adenin und Adenin-*N*-Oxyd treten deutlich hervor, Unterschied gegen ATP und AMP aber undeutlich.
2. Bei dem mit Hydroperoxyd behandelten ATP bzw. AMP treten keine *phosphatfreien* Reaktionsprodukte auf.
3. Die R_f-Werte sind ganz verschieden von den mit der gleichen Flüssigkeit von Bandurski erhaltenen, was offenbar auf der Verwendung eines anderen Papiers (Munktell OB) beruht.

Zwecks Erzielung deutlicherer Ergebnisse wurden schiesslich Versuche angestellt mit dem Entwickler II bei denen aber eine neue Flüssigkeit B, bestehend aus *Wasser-gesättigtem n-Butanol* zur Anwendung kam.

ATP	$R_f = 0$	Adenin	$R_f = 0{,}46$	
H_2O_2 + ATP	$R_f = 0$	Adenin-*N*-Oxyd	$R_f = 0{,}27$	
AMP	$R_f = 0{,}03$	Adenosin	$R_f = 0{,}20$	
H_2O_2 + AMP	$R_f = 0$; $R_f = 0{,}02$		($R_f = 0{,}34$;	sehr kleiner schwacher Flekken; Verunrein.).

Das Chromatogramm mit Flüssigkeit B und Entwickler II zeigt:

1. Sehr deutliche Unterscheidung von Adenin, Adenin-*N*-Oxyd und Adenosin von einander und von ATP und AMP.
2. Beginnende Trennung von AMP und H_2O_2-behandeltem AMP.
3. Bei der H_2O_2 Behandlung von ATP und AMP bildet sich offenbar weder freies Adenin, noch Adenin-*N*-Oxyd, noch Adenosin.

Zur weiteren Trennung von ATP- und AMP-Produkten nach Hydroperoxyd-Behandlung wurde eine spezielle Chromatographie-Flüssigkeit C ausgearbeitet, zusammengesetzt aus 30 Raumteilen Wasser-gesättigtes *n*-Butanol und 7 Raumteilen 98%iger Ameisensäure.

Als Entwickler diente wiederum Entwickler II!

ATP	$R_f = 0$;	($R_f = 0{,}03$, schwacher Flecken; Verunreinigung)
H_2O_2 + ATP	$R_f = 0$;	$R_f = 0{,}07$, starker Flecken; $R_f = 0{,}27$ etwas schwächer
AMP	$R_f = 0{,}23$;	
H_2O_2 + AMP	$R_f = 0{,}23$:	$R_f = 0{,}32$

Das Chromatogramm mit der Flüssigkeit C und dem Entwickler II zeigt:

1. Durch die Hydroperoxyd-Behandlung von ATP entstehen wenigstens zwei Oxydationsprodukte; möglicherweise *N*-Oxyde oder ev. Adenosin-di-phosphorsäure.
2. AMP liefert wenigstens ein Oxydationsprodukt, anscheinend *nicht* identisch mit einem der ATP-Produkte.

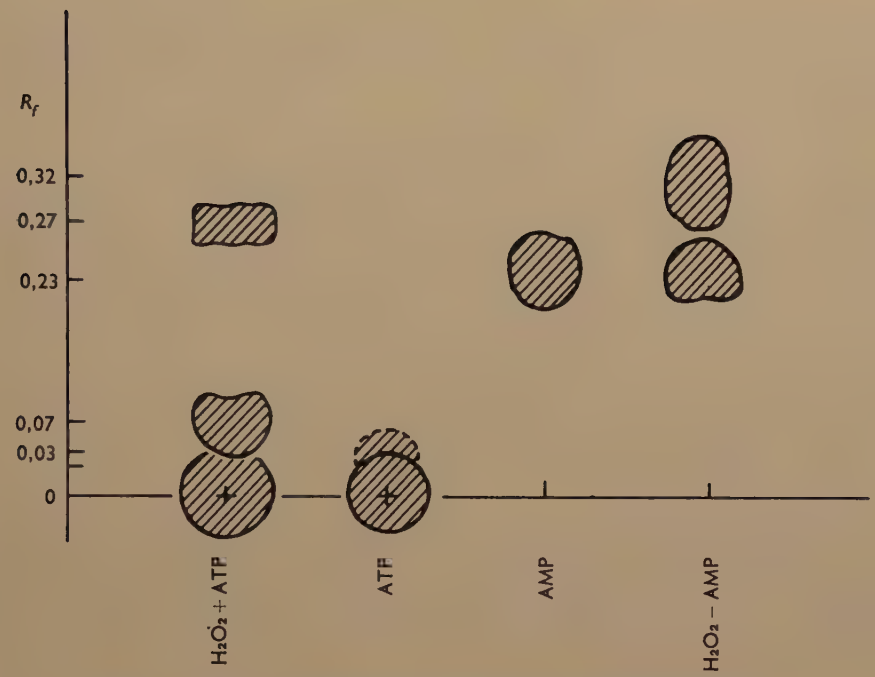

Fig. 2. 30 Tl. W.-gesättigt. n-Butanol; 7 Tl. 98%ig Ameisens.

Ein weiterer analoger Versuch mit Entwickler I (O. Heidenberger) lieferte durchweg Phosphat.

Rolle der Adenosin-triphosphorsäure bei synthetischen Enzymreaktionen

ATP ist als Phosphat-Donator bei Phosphat-Übertragungen imstande, nicht-freiwillig verlaufende Phosphorylierungen und synthetische Enzym-Reaktionen durchzuführen, und ist deshalb einer der wichtigsten Wirkstoffe der enzymatischen Synthese. Diese Fähigkeit der ATP wird stets auf den Energiereichtum dieses Stoffes zurückgeführt. Wir ziehen in Betracht, dass es die an der ATP-Wirkung beteiligten *Enzyme* (Kinasen) sind welche den Energie-Transport vermitteln.

Dadurch ist der Vorgang thermodynamisch ausgedrückt. Eine strukturchemische oder elektronenchemische Beschreibung ist aber damit nicht gegeben.

Bei der Phosphorylierung durch ATP wird der zu übertragende Phosphatrest *in einer energiereichen Form* aus ATP abgespalten und übergeführt. Hierzu ist folgendes zu betonen:

Hydrolytische Spaltungen verlaufen im allgemeinen freiwillig und ohne erhebliche Wärmeentwicklung. Dies ist auch zu erwarten, wenn aus der in der ATP vorliegenden Triphosphorsäure ein Phosphorsäure-Rest als *Anion* abgespalten wird. Die Tendenz der Phosphorsäure zur Esterbildung ist erfahrungsgemäss gering. Wird hingegen die Phosphor-haltige Gruppe als *Kation* abgespalten, so liegt eine sehr viel reaktivere, allerdings kurzlebige Form vor; es besteht dann offenbar eine Analogie zum Verhältnis zwischen Nitrat-Ion und dem sehr viel reaktionsfähigeren Nitronium-Ion, auf das H. v. Euler (11) vor langer Zeit hingewiesen hat und das

von Ingold (12) eingehend entwickelt wurde. Siehe auch H. v. Euler, H. Hasselquist u. I. Loeper, Arkiv Kem. 3, nr 60 (1951). — H. v. Euler, C. r. Acad. Sci. 233, 719 (Paris, 1951).

Porphyrin-N-Oxyde

Umphosphorylierungen von ATP auf Glukose unter Bildung von Hexose-monophosphat und von Adenosin-di-phosphat werden durch ein Enzym bewirkt, welches als Hexosephosphorylase bezeichnet wurde (J. H. Quastel). Dieses von Euler und E. Adler (13) beschriebene Enzym ist in Meyerhofs Hexokinase (14) enthalten.

Das Coenzym, für das Euler und Adler den Namen Co-phosphorylase vorgeschlagen haben, ist offenbar ein Zwischenprodukt von sehr geringen Stabilität, auf das hier nicht weiter eingegangen werden soll.

Der Atmungs-Stoffwechsel führt nach der oxydativen Phosphorylierung über die *Diaphorase* und das Cytochrom-(Keilin)-System, zum Sauerstoff womit die Verbrennung der Nahrungsstoffe vollendet wird.

Es lag deshalb für uns nahe, uns über die Einwirkung von Hydroperoxyd auf Porphyrine zu orientieren.

Durch Papierchromatograhie in Lutidin-Wasser mit als Entwickler ergab sich für Proto-Hämin* der R_f-Wert 0,85, während nach 1-tägiger Behandlung des Hämins bei 20° keine Wanderung, also $R_f = 0$ gefunden wurde, sodass eine starke Veränderung des Hämins eingetreten zu sein schien, u. a. Wohl eine Oxydation der Vinylgruppen.

Herrn Oskar Heidenberger danken wir bestens für wertvolle Mitarbeit, besonders bei der Ausführung der spektrophotometrischen Versuche.

II. Anhang

Pyridoine

Im Anschluss an die beschriebenen Pyridin-*N*-Oxyde und in Hinsicht auf Beziehungen zu Reduktonen haben wir auch Pyridoin-*N*-Oxyde untersucht. Fig. 3 enthält einen spektrophotometrischen Vergleich zwischen 2,2′-Pyridoin und dessen *N*-Oxyd.

2,2′-Pyridoin-N-Oxyd. HH 289.

Zu 0,5 g Pyridoin in 5 ml Eisessig + 1 ml 30%ige Hydroperoxydlös. lässt man 18 Stunden bei 30° stehen. Durch Eindunsten im Vakuum zur Trockne erhielt man ein hellgelbes, allmählich beim Reiben krystallisierendes Öl. Durch Zusatz von wenig Methanol befördert man die Ausscheidung farbloser stäbchenförmiger Krystalle vom Schmpt. 138–139° (ohne Zers.). Bei weiterer wiederholter Krystallisation aus wenig Methanol hielt sich der Schmpt. konstant bei 139,5°. Die Subst. 289 ist schwerlöslich in benzol, löslich in Äthylacetat und Chloroform, leicht löslich in Wasser, Methanol, Äthanol und Aceton.

Analyse: Berechnet für $C_{12}H_{10}N_2O_4$	C 58,53 %	H 4,09 %	N 11,38 %
Gef.	58,81	4,27	11,24

* Wir verdanken das Präparat herrn Professor Dr. Karl Zeile, Fa Boehringer Sohn, Ingelheim/Rhein.

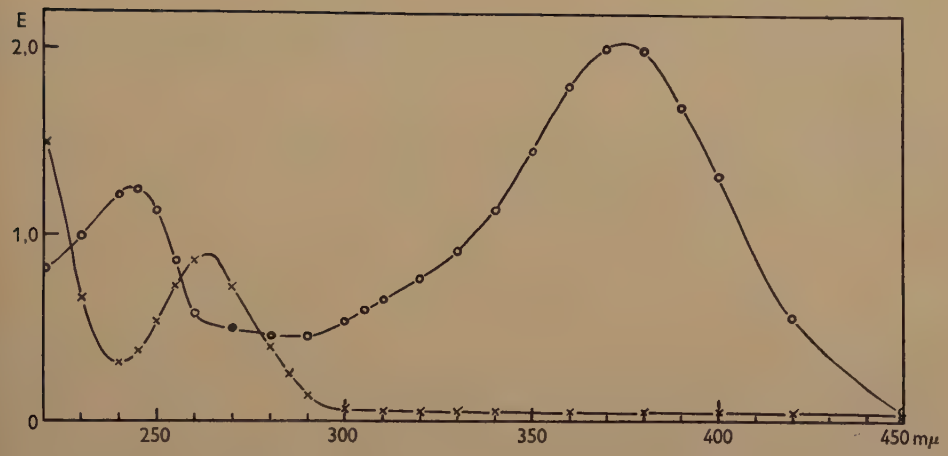

Fig. 3. 33,3 mg/1000 ml A.; –o–o–o– 2,2′-Pyridoin; –×–×–×– 2,2′-Pyridoin-N-oxid (289).

6,6′-Dimethyl-2,2′ pyridoin-N-Oxyd. HH 295.

Zu 1 g Pyridoin in Pulverform + 10 ml Eisessig wurden tropfenweise unter Wasserkühlung 2 ml einer 30%igen Hydroperoxydlösung zugesetzt, wobei die Reaktionstemperatur auf etwa 30° gehalten wurde. Nach 10-stündigem Stehen der Reaktionslösung wurde dieselbe im Vacuum auf ein kleines Volumen eingedunstet. Das dabei erhaltene hellgelbe Öl krystallisierte beim Reiben allmählich; die Krystallmasse wurde mit wenig Isopropanol versetzt und abfiltriert; dieses Rohprodukt (0,6 g) schmolz bei 95–98°.

Durch weiteres zweimaliges Umkrystallisieren aus Isopropanol erhielten wir farblose, gut ausgebildete Stäbchen vom Schmelzpunkt 100,5°. Diese Substanz HH 295 ist in Methanol, Äthanol und Äthylacetat leichtlöslich, in Wasser ziemlich leicht löslich.

Analyse:	Berechnet für $C_{14}H_{14}N_2O_4 + 2\,H_2O$	C	54,19 %	H	5,85 %	N 9,03 %
	Gef.		53,60		5,68	8,78

Das so erhaltene *N*-Oxyd entfärbt — im Gegensatz zum Ausgangsmaterial — TR in saurer Lösung *nicht*. Das 6,6′-Dimethyl-2,2′-pyridoin verhält sich also in dieser Hinsicht ebenso wie das nicht-methylierte Pyridoin. In beiden Fällen wird durch die *N*-Oxydierung die Bildung eines aci-Reduktons gehemmt oder es liegt eine Benzil-Umlagerung vor.

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Structure of laminitol

By Bengt Lindberg and Börje Wickberg

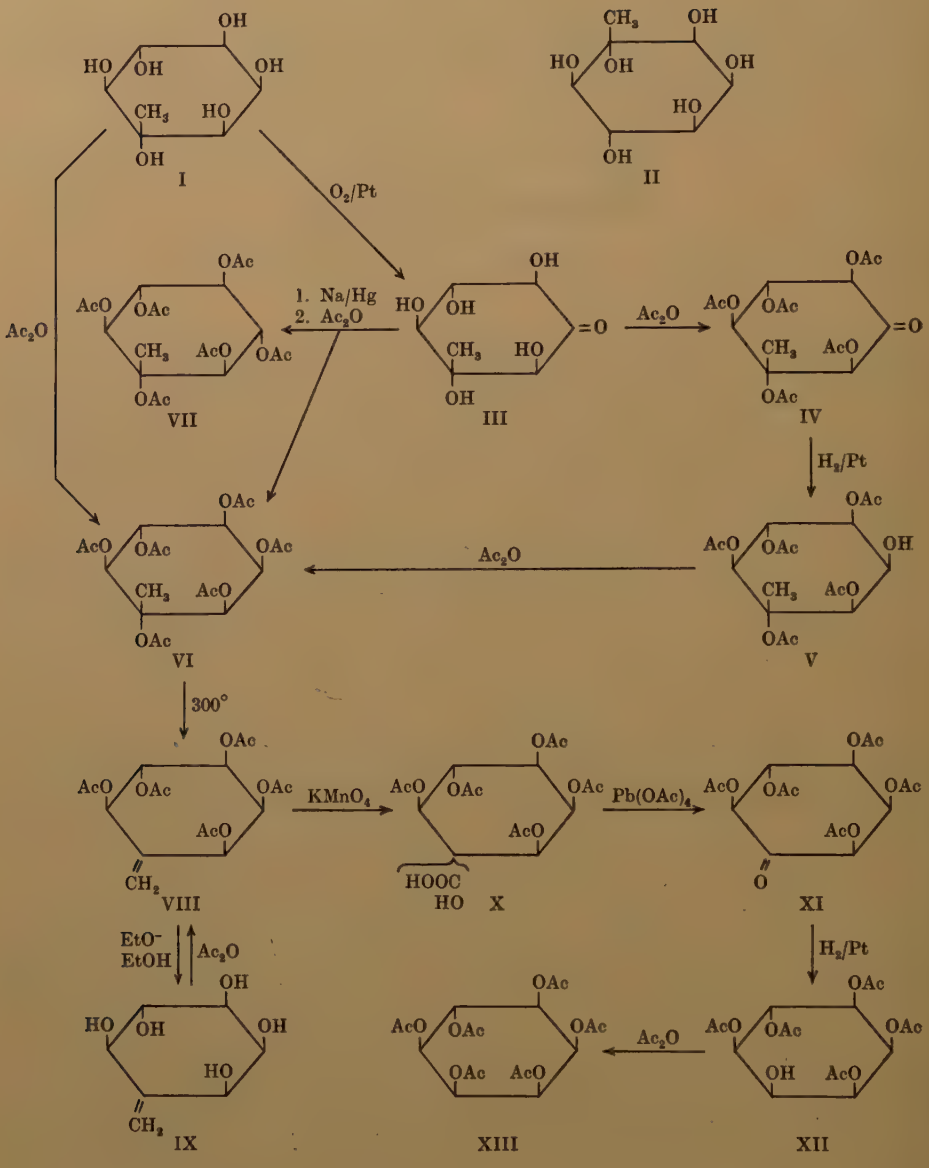
Some years ago a new cyclitol, laminitol, was isolated from the brown alga *Laminaria Cloustoni* (1). A preliminary investigation (2) showed it to be a *C*-methyl-inositol, not identical with mytilitol (3, 4), the only *C*-methyl-inositol previously found in Nature; for several reasons the *myo*inositol configuration was considered the most probable for the new compound. Since then laminitol has also been isolated from other brown algae (5) and from some red algae (6, 7) and is therefore probably a common constituent of these plants.

By a series of reactions described below laminitol has now been shown to be either 4-*C*-methyl-*myo*inositol (I) or its antipode (II). (The nomenclature of Fletcher, Anderson and Lardy (8) is used in the present paper.) It should be emphasised that the work reported here does not decide between the two antipodes; formula I which is used in the following has been chosen arbitrarily and does not necessarily represent the absolute configuration of laminitol.

The dehydrogenation of polyols by treatment with oxygen in the presence of platinum catalysts has been studied by Heyns and coworkers and a summary of this field has recently been published by Heyns and Paulsen (9). In the cyclitol series it has been found that axial hydroxyl groups are preferentially dehydrogenated; this reaction is reversible as axial hydroxyl groups are formed by platinum catalysed hydrogenation of the corresponding ketones. Reduction with sodium amalgam, on the other hand, is known to give an epimeric pair of inositols (10).

When laminitol was treated with oxygen over Adams' catalyst it gave a complex mixture of products, containing two main constituents which had the reducing properties of inososes. One of them was formed in relatively low yield (9 %) and did not crystallise. After hydrogenation over platinum catalyst it gave two strong spots on paper chromatograms which, in view of the behaviour of other cyclitols, would indicate that the inosose-like compound had not been formed by normal attack on an axial hydroxyl group. This fraction was therefore discarded. The other component (III), obtained in 35 % yield, was crystalline, m.p.[1] 157–164° (decomp.), $[\alpha]_D$ −15°. It analysed for a *C*-methyl-inosose and on acetylation gave a pentaacetate (IV), melting at 148–153° (decomp. uncorr.) in a Pyrex capillary tube. The inosose acetate was quite labile, which seems to be a characteristic property of inosose acetates in general, and considerable losses were incurred in purification. Catalytic hydrogenation of IV gave a chromatographically homogeneous product (V) from which laminitol hexaacetate (VI) was obtained by acetylation. This result strongly indicates that III was formed in the expected way by dehydrogenation at an axial hydroxyl group in laminitol.

[1] Melting points are corrected unless stated otherwise.



When III was reduced with sodium amalgam at pH 6, a mixture of cyclitols was obtained. The mixture was acetylated and separated on a dimethyl sulphoxide-treated silica gel column, giving the hexaacetates of laminitol and mytilitol (VI and VII, resp.), indistinguishable from authentic specimens. These experiments establish the *myo*inositol configuration of laminitol.

The substitution of hydrogen for the *C*-methyl group in laminitol accompanied by inversion would give an inositol the configuration of which would determine the

position of the *C*-methyl group. Bailey *et al.* have investigated the thermal elimination of acetic acid from tertiary methylcyclohexyl acetates with the formation of the corresponding methylenecyclohexane (11) and they have shown that negative substituents in the β-position do not change the course of elimination reactions (12) of this type. Pyrolysis of laminitol hexaacetate (VI) at 300° gave the pentaacetate of a methylene*cyclo*hexanepentaol (VIII) in about 40 % yield. It was deacetylated to the crystalline pentaol (IX), m.p. 207–208°, $[\alpha]_D$ +24°, this was reacetylated and the acetate (VIII) was oxidised with potassium permanganate in acetic acid. A synthetic isomer of VIII has been oxidised in a similar manner by Posternak and Reymond (13) who showed that the product was an α-hydroxy acid. The acid expected (X) was not isolated; the product was instead immediately oxidised further with lead tetraacetate to an inosose pentaacetate (XI), chromatographically indistinguishable from *epi*inosose-2 pentaacetate. Catalytic hydrogenation of XI gave an inositol pentaacetate (XII) which was converted to a hexaacetate, which was identical with authentic *epi*inositol hexaacetate (XIII). This shows that an axial hydroxyl group was formed in the hydrogenation of XI, and consequently proves structure I for laminitol.

The inosose pentaacetate XI is either D- or L-*epi*inosose-2 pentaacetate. D-*epi*-Inosose-2 has been prepared by biochemical oxidation of *epi*inositol (14) and has been characterised as the crystalline pentabenzoate. This optically active inosose is obviously a key substance in the assignment of an absolute configuration to laminitol.

The reactions were all followed by paper chromatography. For the polyols, aqueous acetone was used as eluent; the acetylated products were run on dimethyl sulphoxide-impregnated paper with a lipophilic solvent such as light petroleum or *iso*propyl ether as the mobile phase (15). Separation of cyclitol acetates on a preparative scale was done on columns of silica gel with dimethyl sulphoxide as the stationary phase. This promises to be a fractionation method of general value.

The recent isolation of both mytilitol and laminitol from the red alga *Polysiphonia fastigiata* (7) is of special interest as it supports a previous suggestion (2) that these substances could be biochemically interconvertible *via* oxidation—reduction reactions analogous to those described above.

Experimental

Paper chromatography

Paper chromatograms of the free cyclitols were eluted with 80 % aqueous acetone or, in the case of the inososes, with acetone–10 % aqueous acetic acid (4:1). Acetylated derivatives were separated as described in a previous paper (15), using Whatman No. 1 filter paper impregnated with dimethyl sulphoxide (abbrev. DMSO) and either ethyl ether, *iso*propyl ether or light petroleum as mobile phase. When using ethyl ether as the eluent the papers were impregnated in the usual way with DMSO in toluene and then dried at room temperature; these papers contained considerable amounts of absorbed moisture ("moist papers"). Spots were generally detected by spraying with silver nitrate–sodium ethoxide (15, 16). It was found that inososes and their acetates, when run on unwashed paper, could be detected as pink spots if the paper after heating at 130° for 5 min was sprayed with aqueous silver nitrate solution and then dried. This method seemed to be quite sensitive but was probably dependent on the presence of impurities in the paper as the sensitivity became unsatisfactory when the papers were prewashed with water and butanol.

Column chromatography

Dry packed columns of Whatman ashless cellulose powder as described by Anderson *et al.* (17) were used with aqueous acetone as solvent.

Preliminary experiments had shown that with DMSO–*iso*propyl ether as solvents the acetylated sugars travelled in much the same way on columns of silica gel as on filter paper; columns of this type were used for the separation of acetylated inositol derivatives. Silica gel (Gebr. Herrmann, Köln-Ehrenfeld; 0.04–0.09 mm) was mixed with a 5 % v/v solution of DMSO in chloroform. The suspension was kept for a short time in an evacuated vacuum desiccator to remove air bubbles and was then filled into a separating funnel attached to the top of a chromatogram tube. This had previously been filled with enough of the same chloroform solution to bring the meniscus of the liquid above the stopcock of the separating funnel. The mouth of the separating funnel was then connected with a flask filled with air under moderate pressure, and the stopcocks were opened so that the silica gel was packed into the chromatogram tube in a strong current of solvent. It was found that tapping the column during the sedimentation process or later would cause an uneven flow through the column, and this was therefore protected from mechanical vibration. The column was washed with at least 5 column volumes of an ethyl ether–DMSO mixture (1 part of dry ethyl ether + 4 parts of ethyl ether which had previously been equilibrated towards an excess of DMSO containing 4 % v/v of water), followed by a small quantity of dry ethyl ether and finally with the *iso*propyl ether–DMSO mixture (2 column volumes, prepared in the same way as the ethyl ether mixture above) which was also used as the eluent. Slightly moist ether solutions of DMSO were used rather than anhydrous, saturated solutions since absorption of even traces of atmospheric moisture by the latter would cause the precipitation of a DMSO phase.

Isolation and further characterisation of laminitol

The brown alga *Laminaria Cloustoni* (12 kg) was worked up as described in a previous paper (1); repeated fractionation on carbon columns finally gave pure laminitol (6 g), m.p. 266–269°, $[\alpha]_D^{20}$ −3° (water, $c = 2$).

The electrophoretic mobility of laminitol in borate buffer at pH 10, relative to D-glucose, was 0.83. The corresponding values for *myo*inositol and *iso*mytilitol (2-*C*-methyl-*myo*inositol) were 0.45 and 0.08, respectively.

The infrared spectrum of laminitol (KBr disc) showed a strong absorption peak at 958 cm^{-1} which compares fairly well with the corresponding value (963 cm^{-1}) found for mytilitol and *iso*mytilitol by Barker *et al.* (18), who assigned this band to a *C*-methyl rocking mode.

4-C-*Methyl*-myo*inosose*-2 (*III*)

A stream of hydrogen was passed through a suspension of platinum dioxide (90 mg) in water (10 ml) in a 100 ml Kluyver flask until reduction was complete. Excess hydrogen was removed by evacuating the flask and then passing a steady stream of oxygen through it until the metal was finely suspended; laminitol (191 mg) was then added and oxidised with a stream of oxygen at atmospheric pressure for 2.5 h at 20°.

On paper chromatograms run with the acetone–aqueous acetic acid mixture the oxidised solution gave a strong spot with the R_F value (0.10) of laminitol and two spots with R_F 0.18 (strong) and 0.23 (weak) which gave a colour reaction for inososes with silver nitrate. Some fast and very trailing spots were also present. Chromato-

grams of samples withdrawn at an early stage of the reaction indicated that the oxidation products were formed in about the same proportions from the beginning. The oxidised solution was filtered and the filtrate was concentrated under reduced pressure, giving a residue which crystallised spontaneously. Recrystallisation from aqueous ethanol gave laminitol (79 mg). The mother liquors were combined with the amorphous product obtained by oxidising a second lot of laminitol (180 mg) for 2 h at 30° in the same way and the combined material was extracted with acetone–10 % aqueous acetic acid (4:1; 3 × 5 ml). The extracts were added successively to the top of a cellulose column (2.3 × 22 cm) which was then eluted with the same solvent. Chromatograms of the eluate showed that the remaining laminitol and the two inosose components were almost completely separated but were all contaminated with material giving faint and very trailing spots. The appropriate fractions were concentrated at 30° and 15 mm pressure.

The laminitol fraction (63 mg) gave crystalline laminitol (55 mg) from methanol–acetone.

The inosose fraction first eluted (28 mg) gave a single main spot with R_F 0.23; it was not obtained in a crystalline state.

After an intermediate fraction (10 mg) which gave both inosose spots (R_F 0.18 and 0.23) and was discarded, a second inosose fraction (120 mg in total) was obtained which gave one predominant spot with R_F 0.18 on paper chromatograms. To facilitate crystallisation this fraction was collected in two lots, one from the most concentrated part of the eluate (90 mg) and the other (30 mg) containing the remainder. When dissolved in a mixture of methanol and ethanol containing a trace of water, the main part of this fraction slowly crystallised (30 mg), mp. 158–166° (loss of solvent of crystallisation at 100–110°). A further quantity (20 mg) of crystalline material was obtained by separating the remaining amorphous material on a cellulose column as described above. The combined crystalline product was purified by treatment with decolorising carbon in methanol–ethanol and recrystallised from the same (anhydrous) solvent. This gave 4-*C*-methyl-*myo*inosose-2 (III) in a solvent-free state, m.p. 157–164° (decomp.), $[\alpha]_D^{19}$ −15° (water, $c = 1.2$). (Found: C 43.6; H 6.12. Calc. for $C_7H_{12}O_6$: C 43.8; H 6.29.)

4-*C*-Methyl-*myo*inosose-2 pentaacetate (IV) was prepared by treating III (15 mg) with acetic anhydride containing 0.3 % of perchloric acid (0.3 ml) for 1.5 h at room temperature and then adding an excess of water. After decomposition of excess anhydride the mixture, which contained crystals, was extracted with chloroform, the extract was washed repeatedly with water, concentrated under reduced pressure to a small volume and then diluted with light petroleum. The crude crystalline product (30 mg), m.p. 120–129° (decomp.), was recrystallized from aqueous methanol, carbon tetrachloride–light petroleum (1:1) and methanol. The purified acetate (19 mg) had m.p. *ca* 123–135° (decomp.) when determined on the micro hot stage and 148–153° (decomp., uncorr.) in a Pyrex capillary tube. (Found: C 51.6; H 5.60. Calc. for $C_{17}H_{22}O_{11}$: C 50.7; H 5.51.)

The difference in the melting points recalls the observations on *myo*inosose-2 pentaacetate where the alkaline soda-glass has been shown to cause decomposition during m.p. determination (19).

When laminitol was oxidised catalytically under slightly more vigorous conditions (3 h at 45° or 2 h at 40°) and the product was separated as described above less laminitol was recovered but the yields of the amorphous inosose fractions were practically the same. The fraction giving the inosose spot of R_F 0.23 after hydrogena-

tion in aqueous solution over Adams' catalyst gave two equally strong spots on paper chromatograms; one had the R_F value of laminitol (0.10) while the other was much faster (R_F 0.20). A sample of the crude amorphous 4-*C*-methyl-*myo*inosose-2 after hydrogenation over platinum gave the single strong spot expected (R_F value as laminitol) with only traces of a faster component; after acetylation the reduced material was chromatographically indistinguisable from laminitol hexaacetate.

*Laminitol hexaacetate (VI) from 4-*C*-methyl-myo*inosose-2 pentaacetate (IV)*

A mixture of 4-*C*-methyl-*myo*inosose-2 pentaacetate (8 mg) and Adams' catalyst (4 mg) in ethanol (2 ml) containing acetic acid (1 drop) was shaken with hydrogen for 3 h at room temperature and atmospheric pressure. Concentration of the filtered solution gave amorphous laminitol pentaacetate (V) which on DMSO-treated paper chromatograms gave a single spot, R_F *ca* 0.05 and 0.28 with *iso*propyl ether (paper dried at 60°) and ethyl ether (moist paper), respectively, as eluents. The pentaacetate was acetylated with acetic anhydride containing 0.3 % of perchloric acid and the crude product, isolated in the usual way, was recrystallised from carbon tetrachloride–light petroleum to give laminitol hexaacetate (6 mg), m.p. and mixed m.p. 156–158°.

*Sodium amalgam reduction of 4-*C*-methyl-myo*inosose-2 (III)*

Amorphous but chromatographically pure 4-*C*-methyl-*myo*inosose-2 (150 mg) was dissolved in 0.6 *M* sodium succinate buffer (pH 6.0; 6 ml); mercury (1 g) and 2.5 % sodium amalgam (0.5 g portions, 8 g in total) were then added with vigorous stirring while the pH of the solution was kept at 6.0 ± 0.5 by adding 4 *M* acetic acid. The resultant solution no longer reduced Fehling solution; it was deionised with Amberlite resins IR 120 and IR 4B and then concentrated under reduced pressure giving a residue which crystallised spontaneously and on paper chromatograms gave a somewhat elongated spot with R_F value about the same as laminitol and mytilitol (0.10 and 0.09, respectively). The mixture was treated with acetic anhydride containing 0.3 % of perchloric acid and the acetylated product was isolated in the usual way as a syrup (300 mg) which on DMSO-treated paper chromatograms run in *iso*propyl ether gave one strong spot and one fainter spot with the R_F values of the hexaacetates of laminitol and mytilitol (0.26 and 0.19, respectively).

The acetate mixture was dissolved in *iso*propyl ether (20 ml) containing some ethyl ether (*ca* 3 ml) and this solution was added to the top of a DMSO-saturated silica gel column which was then eluted (rate 1.5 ml/min) with *iso*propyl ether containing moist DMSO (see above). The eluate was collected in 13 ml lots which after examination by paper chromatography were combined giving three main fractions. These were concentrated, the residues were dissolved in chloroform and the chloroform solutions were washed with water to remove DMSO, dried over sodium sulphate and concentrated to dryness *in vacuo*.

The first fraction (160 mg) gave crystals (140 mg), m.p. 155–157°; recrystallisation from aqueous methanol gave pure laminitol hexaacetate, m.p. and mixed m.p. 156–158°.

An intermediate fraction (8 mg) gave spots for both components and was discarded.

The third fraction (60 mg) was recrystallised from carbon tetrachloride–light petroleum and then from methanol to give mytilitol hexaacetate which is dimorphous, m.p. and mixed m.p. 172–174° (first modification) and 184–187° (second modification).

The above result was checked chromatographically starting from a small sample of

crystalline 4-*C*-methyl-*myo*inosose-2 which was reduced with sodium amalgam at pH 6. Paper chromatograms of the mixture of *C*-methyl-inositols formed and of the acetylated mixture agreed completely with those from the large scale experiment.

Pyrolysis of laminitol hexaacetate.
4-Methylene-4-deoxy-myo*inositol* (*IX*)

Laminitol hexaacetate (199 mg) was filled into a Pyrex test tube (0.8 × 24 cm) and then covered with a 10 cm layer of crushed porcelain which had previously been dried *in vacuo* at 300°. The tube was inserted in a hole drilled through an aluminium block (*ca* 12 cm wide), heated at 300°, leaving 2 cm of the bottom part of the tube outside the block. After 10 min the tube was moved so as to heat its entire lower part; a colorless distillate with a strong smell of acetic acid collected in the cold part of the tube. After a further 8 min the tube was evacuated for 20 min with a water pump and the viscous liquid (170 mg) which had distilled over was examined by paper chromatography in DMSO-*iso*propyl ether. The silver nitrate–sodium ethoxide spray showed a strong spot with R_F 0.16 and faint spots of laminitol hexaacetate (R_F 0.27) and unknown compounds (R_F *ca* 0.33 and 0.50).

The pyrolysate was separated on a silica gel column (1.1 × 12 cm) with DMSO–*iso*propyl ether as solvents, essentially as described above for the separation of laminitol and mytilitol hexaacetates. It was eluted at a rate of 1 ml/min and the eluate was collected in 4.5 ml fractions which were examined by spot tests and paper chromatography. Appropriate fractions were combined and worked up as described previously.

The first fractions contained small quantities of a large number of substances. Fractions 18–21 (11 mg) gave essentially a single spot with the R_F value of laminitol hexaacetate but reacting like an inosose acetate with silver nitrate. Fractions 22–26 (16 mg) were chromatographically homogeneous and gave crystalline laminitol hexaacetate. Fractions 32–50 gave 4-methylene-4-deoxy-*myo*inositol pentaacetate (VIII) as a syrup (75 mg) which gave only one spot on chromatograms run in *iso*-propyl ether (R_F 0.16) or light petroleum (R_{GA}[1] 0.45). It was warmed slightly for a few minutes with absolute ethanol (3 ml) containing catalytic amounts of sodium ethoxide, and after dilution with water the solution was passed through columns of Amberlite resins IR 120 and IR 4B and then concentrated to dryness under reduced pressure. The residue, which contained traces of silicone grease was dissolved in water and the solution was filtered and then taken to dryness. The slightly coloured residue (36 mg) crystallised spontaneously and was recrystallised by adding ethanol to a concentrated aqueous solution, yielding 4-methylene-4-deoxy-*myo*inositol (IX) (30 mg or 38 % calc. on laminitol hexaacetate), m.p. 205–208°. It was sublimed (180° bath temperature and 0.1 mm) and recrystallised from aqueous ethanol; m.p. 207–208°, $[\alpha]_D^{21}$ +24° (water, $c = 1.9$). (Found: C 48.2; H 7.00. Calc. for $C_7H_{12}O_5$: C 47.7; H 6.87.)

It decolorised bromine water immediately. On paper chromatograms run in aqueous acetone it gave a spot with R_F 0.25.

epi*Inositol hexaacetate* (*XIII*) *from 4-methylene-4-deoxy*-myo*inositol* (*IX*)

4-Methylene-4-deoxy-*myo*inositol (16.3 mg) was treated for a few hours at room temperature with acetic anhydride containing 0.3 % of perchloric acid. The excess anhydride was then decomposed with water and the mixture was washed with

[1] Mobility relative to 1,2,3,4,6-penta-*O*-acetyl-α-D-glucose (15).

sodium bicarbonate and water, dried and concentrated to give the desired 4-methylene-4-deoxy-*myo*inositol pentaacetate (VIII) as a syrup (37 mg). On paper chromatograms it was indistinguishable from the product obtained by pyrolysing laminitol hexaacetate.

A solution of 4-methylene-4-deoxy-*myo*inositol pentaacetate (37 mg, 93 μmole) was dissolved in permanganate-treated, redistilled acetic acid (10 ml), and an acetic acid solution of potassium permanganate (3.2 mg or 20 μmole of oxidant/ml) was then added in 1 ml portions. Each portion was added only after the colour of the preceding one had faded. The reaction was followed by chromatography on DMSO-impregnated paper equilibrated with atmospheric moisture with *iso*propyl ether as eluent; the solvent front was allowed to travel *ca* 10 cm. 10 μl samples of the reaction mixture were withdrawn and after addition of a few μl of ethanol they were concentrated to dryness in a current of air with slight warming; the residual material was extracted with ethyl ether and this solution was applied quantitatively to the paper chromatograms. As the oxidation proceeded, a very slow spot (R_F value *ca* 0.1 of that of the starting material) appeared, probably from an α-hydroxy acid (X), and towards the end of the reaction indications were also obtained of the formation of rather small amounts of a compound travelling with about half the speed of the starting material and chromatographically very similar to *epi*inosose-2 pentaacetate. This was thought to be D- or L-*epi*inosose-2 pentaacetate (XI) formed by further oxidation of X. After the addition of 9 ml of the permanganate solution (approximately the theoretical amount required) no more starting material could be detected on paper chromatograms. When these were run in ethyl ether and then sprayed with an indicator solution (20) the oxidised material gave three spots, R_F 0.30 (faint, probably acetic acid), R_F 0.45 (strong) and R_F 0.70 (faint). The same chromatogram was then dried for 5 min at 120° and sprayed with silver nitrate and sodium ethoxide; only the spot with R_F 0.45 had a reducing action.

Some sulphur dioxide was passed into the oxidation mixture which was heated slightly (*ca* 40°). After a few minutes when the brown colour had disappeared the turbid solution was concentrated under reduced pressure to about half its original volume. The precipitate was removed by centrifuging and washed with acetic acid, the solution and washings were combined (total volume *ca* 20 ml) and diluted with water (2 ml). A solution of lead tetraacetate (3.5 ml; 8.8 mg or 20 μmole of oxidant/ml) was added in 0.5 ml portions and the reaction was followed by paper chromatography as described above. As expected the slow spot of the assumed α-hydroxy acid gradually disappeared while the inosose spot grew stronger. 1 h after adding the last portion of lead tetraacetate, sulphur dioxide was passed into the reaction mixture to reduce the remaining oxidant. The turbid solution was concentrated to a small volume under reduced pressure, the residue was shaken with a water–chloroform mixture and after filtration the chloroform phase was washed with water and concentrated to dryness under reduced pressure to give a thick syrup (31 mg), presumably D- or L-*epi*inosose-2 pentaacetate (XI).

The product from the lead tetraacetate oxidation (30 mg) was dissolved in absolute ethanol (3 ml) containing acetic acid (1 drop) and was then hydrogenated (3 h) at room temperature and ordinary pressure in the presence of Adams' catalyst (11 mg). After filtration the solution was concentrated to dryness under reduced pressure giving a syrup (27 mg) which was presumably the *epi*inositol pentaacetate XII. On chromatograms on DMSO-treated paper it gave only a single spot, R_F *ca* 0.01 and 0.14 with *iso*propyl ether and ethyl ether, respectively.

Racemic *epi*inosose-2, after hydrogenation as above, gave identical chromatograms.

The *epi*inositol pentaacetate (26 mg) was treated for 2 h at room temperature with acetic anhydride containing 0.3 % of perchloric acid and the product was isolated in the usual way. The syrup obtained (26 mg) gave crystals from chloroform–light petroleum, m.p. 189–192°. Recrystallisation from aqueous methanol gave pure *epi*-inositol hexaacetate (XIII) (20 mg), m.p. 190–192° alone or mixed with an authentic specimen. Infrared spectra confirmed its identity.

SUMMARY

Laminitol, a *C*-methyl-inositol isolated from various brown and red algae, has now been shown to be either 4-*C*-methyl-*myo*inositol (I) or its antipode (II) by the following evidence.

1. Catalytic dehydrogenation of laminitol gives a *C*-methyl-inosose (III) which on reduction with sodium amalgam gives a mixture of laminitol and mytilitol, proving the *myo*inositol configuration of laminitol.

2. Thermal elimination of acetic acid from laminitol hexa-acetate gives the *exo*methylene compound VIII which can be degraded to the inosose pentaacetate XI. The latter gives *epi*inositol hexaacetate (XIII) on catalytic hydrogenation and subsequent acetylation. This proves the position of the methyl group of laminitol.

The preparation of dimethyl sulphoxide-treated silica gel columns for the separation of acetylated inositol derivatives is described.

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Umsatz der Trehalose in Bäckerhefe

II. Die Synthese von Trehalosemonophosphat und Trehalose in verschiedenen Gärungssystemen aus Brauerei- und Bäckerhefe

Von Majken Elander

Mit 2 Figuren im Text

In einer früher erschienenen Untersuchung über die Trehalosebildung bei der Vergärung von Glucose mit acetongetrockneter Bäckerhefe (1) konnte gezeigt werden, dass unter geeigneten Bedingungen 20–30 % der zugesetzten Glucose in Trehalose umgewandelt wurden. Bei dieser Untersuchung wurden Versuche angestellt, THP[1] gleichzeitig mit HMP auszufällen. Wenn der Ester überhaupt vorkam, war aber die Menge so niedrig, dass sie zufolge der Einwirkung von HMP weder aus der optischen Aktivität noch aus der Zunahme des Reduktionswertes bei der Säurehydrolyse mit Sicherheit bestimmt werden konnte.

Bei der Vergärung von Glucose mit getrockneter englischer Brauereioberhefe (2) und mit frischer Brauereiunterhefe in Gegenwart von Toluol (3) ist THP isoliert worden. Es ist auch gezeigt worden, dass mit einem gereinigten Enzym aus Brauereihefe THP aus UDPG und G-6-P synthetisiert wird (4, 5). Im gereinigten Enzym kam eine Phosphatase vor, die spezifisch auf THP wirkte. Die Annahme, dass THP ein Zwischenprodukt bei der Synthese von Trehalose in *Saccharomyces cerevisiae* sei, ist demnach gut begründet.

Dass THP in Gärungen mit acetongetrockneter Bäckerhefe nicht nachgewiesen werden konnte, könnte darauf beruhen, dass dieser Ester in Bäckerhefe bedeutend schneller als in Brauereihefe dephosphoryliert wird. In Anbetracht, dass der endogene Gehalt an Trehalose der Bäckerhefe meistens demjenigen der Brauereihefe weitaus übertrifft (6, 7), erscheint diese Annahme wohl begründet zu sein.

In der vorliegenden Untersuchung sind die Mengen von THP und Trehalose verglichen, die bei der Vergärung von Glucose mit getrockneter Brauereihefe und Toluol einerseits und mit einigen Präparaten aus Bäckerhefe anderseits gebildet wurden. THP wurde, wenn möglich, als Bariumsalz mit Alkohol gefällt. THP und Trehalose wurden sowohl aus der optischen Aktivität wie aus der Zunahme des Reduktionswertes bei der Säurehydrolyse bestimmt.

THP konnte aus Gärproben mit getrockneter Brauereihefe und Toluol isoliert

[1] THP = Trehalosemonophosphorsäure; HMP = Hexosemonophosphorsäure; UDPG = Uridindiphosphoglucose; G-6-P = Glucose-6-phosphorsäure; HDP = Hexosediphosphorsäure; G-1-P = Glucose-1-phosphorsäure; F-6-P = Fructose-6-phosphorsäure; F-1-P = Fructose-1-phosphorsäure; F-1,6-P = Fructose-1,6-diphosphorsäure; UDP = Uridindiphosphorsäure.

werden. Mit Hilfe dieses Esters als Vergleichssubstanz konnte ein Lösungsmittel (8) ausprobiert werden, das bei der Papierchromatographie eine gute Trennung von THP und HMP bewirkte. Mit dieser Methode war der sichere Nachweis von THP möglich, auch wenn die Menge so niedrig war, dass die oben erwähnten Analysemethoden unzuverlässige Werte gaben.

Experimenteller Teil

Getrocknete Brauerei- und Bäckerhefe wurden nach Nilsson (9) hergestellt. Zur Herstellung der getrockneten und der acetonbehandelten Bäckerhefe wurde Hefe aus derselben Lieferung genommen. Durch Gefriertrocknung der gewaschenen Acetonhefe wurde ein Hefepräparat erhalten, das im Vergleich mit der vorher auf Tonplatten getrocknete Hefe eine erheblich grössere Gärungsaktivität besass.

Lipmann (10) hat eine Methodik angegeben, um aus autolysierter Bäckerhefe gärungsaktive Extrakte zu erhalten. Wenn die schwedische Bäckerhefe nach dieser Methode behandelt wurde, zeigten die Extrakte fast ausnahmslos ein schlechtes Gärvermögen. Wahrscheinlich trocknete die Hefe zu langsam, so dass eine teilweise Inaktivierung der Fermente eintrat. Deswegen wurde die Hauptmenge des Wassers vor der Autolyse aus der Hefe abgepresst. Danach wurde sie durch ein Sieb getrieben und in flachen Schalen in 1–2 cm hoher Schicht getrocknet. Zwei in verschiedener Weise autolysierte Hefepräparate sind in dieser Untersuchung verwendet worden. Das eine Präparat wurde 4 Tage bei 19°–22° im Thermostat langsam getrocknet (Hefe A). Bei dem anderen (Hefe B) dauerte das Trocknen nur 24 Stunden und wurde offen bei 27°–29° vorgenommen. Die beiden autolysierten Hefen wurden danach 3 Tage im Vakuumexsiccator über Blaugel und konz. Schwefelsäure getrocknet. Zur Herstellung der Extrakte wurde die autolysierte Hefe 2 Stunden mit 3 Teilen 0,17 M sek. Natriumphosphatlösung bei 38° digeriert.

Die Gärversuche wurden wie früher ausgeführt (1). Die Gärproben mit getrockneter Brauereihefe hatten folgende Zusammensetzung; 200 mg Trockenhefe, 100 mg Glucose, 0,1 ml 1,1 M Phosphatpuffer (pH 6,4), 0,03 ml Hexosediphosphatlösung (14,5 mg org. Phosphat/ml), 0,03 ml 2%ige Acetaldehydlösung, dest. Wasser ad 2 ml. 0,2 ml Toluol wurden pro Gärprobe zugesetzt. In den Gärproben mit getrockneter Bäckerhefe wurden statt 0,1 ml 1,1 M Phosphatpuffer 0,2 ml 0,5 M sek. Natriumphosphat zugefügt. Die Gärproben mit acetongetrockneter und gewaschener Bäckerhefe wurden wie die Proben mit getrockneter Bäckerhefe zusammengesetzt. 0,5 ml Hefekochsaft (ein Teil getrockneter Brauereihefe und drei Teile Wasser) und 0,1 ml 0,2 M Magnesiumchlorid wurden aber diesen Proben zugefügt. Diese Gärversuche wurden ohne und mit Toluol ausgeführt. Die Beendigung der Glucosegärung war mit der acetongetrockneten Bäckerhefe manchmal schwieriger zu beurteilen als diejenige der übrigen Hefepräparate, weil die Vergärung der endogenen Kohlehydrate lebhafter einsetzte. Durch Vergärung mit frischer Bäckerhefe konnte aber die in den Hefefiltraten eventuell vorhandene freie Glucose bestimmt werden. 0,5 ml Hefesuspension (1 g 25%ige Bäckerhefe in 0,5 ml dest. Wasser) wurden pro Gärprobe zugesetzt. Die Gärversuche mit Extrakten aus autolysierter Bäckerhefe wurden wie die Proben mit getrockneter Brauereihefe zusammengesetzt; statt 200 mg Trockenhefe wurde aber 1 ml Hefeextrakt zugesetzt. Die Gärungen wurden ohne Toluol ausgeführt.

Da die Permeabilität der in dieser Untersuchung vorkommenden Hefepräparate

verschieden war, mussten sowohl die Nullproben wie die Gärproben in verschiedener Weise für die Analysen vorbereitet werden. Die in dünnen Schichten bei Zimmertemperatur schnell getrocknete Brauereihefe ist für Phosphorsäureester leicht permeabel (9, 11). Wenn die getrocknete Brauereihefe in Gegenwart von Phosphatpuffer und Toluol einige Stunden lang im Thermostat bei 30° geschüttelt wurde, wurde die endogene Trehalose ziemlich schnell vergoren. Die Extraktion der Hefetrehalose musste deswegen so unternommen werden, dass keine Vergärung des Zuckers stattfinden konnte. Drei verschiedene Extraktionsversuche wurden angestellt: a) 2 g getrocknete Brauereihefe wurden dreimal 15 Min. lang mit je 10 ml dest. Wasser bei Zimmertemperatur extrahiert. Die Analysen wurden in den vereinigten Zentrifugaten ausgeführt. b) und c) Dieselbe Hefemenge wurde dreimal während einer Stunde bei 3° mit je 10 ml 5%iger Trichloressigsäure bzw. 7 %iger Überchlorsäure extrahiert.

Aus der getrockneten Bäckerhefe lassen sich die Phosphorsäureester mit Wasser nicht vollständig auswaschen. Die Form der Gärkurve wird auch durch Phosphatzusatz weniger beeinflusst als diejenige der Brauereihefe (12). In Gegenwart von Toluol wurde bei 30° die endogene Trehalose der Bäckerhefe wie diejenige der Brauereihefe schnell vergoren. Die Nullproben der Bäckerhefe wurden deswegen durch Extraktion mit 7%iger Überchlorsäure bei 3° hergestellt. 2 g Hefe wurden dreimal während einer Stunde mit je 10 ml Säure extrahiert. Da die Überchlorsäure nicht wie die Trichloressigsäure bei den Zuckerbestimmungen einwirkt (13), wurde diese Säure als Extraktionsmittel vorgezogen.

Aus der acetongetrockneten und gewaschenen Bäckerhefe können Phosphorsäureester und Zucker mit Wasser vollständig entfernt werden. Dieselben Trehalosewerte wurden erhalten, wenn die Extraktion mit Säure oder mit Wasser ausgeführt wurde (nicht veröffentlicht). Phosphorsäureester und Zucker wurden sowohl in der Hefe als auch im Kochsaft bestimmt. 2 g Acetonhefe wurden dreimal mit je 10 ml dest. Wasser 15 Min. lang bei Zimmertemperatur extrahiert. Wenn kein Hefekochsaft vorhanden war, fand keine Vergärung der endogenen Trehalose statt.

Wenn die Gärungen beendigt werden sollten, wurden die Proben im Eiswasser abgekühlt und die Hefe abzentrifugiert. In den Gärproben mit getrockneter Brauereihefe und Toluol und in denjenigen mit Acetonhefe, wo die Hefe für Phosphorsäureester und Trehalose vollständig permeabel war, wurden die Analysen direkt im Zentrifugat ausgeführt. Die Proteine wurden mit 30%igem Quecksilbersulfat in 10%iger Schwefelsäure gefällt, und die Proben dann mit festem Bariumkarbonat neutralisiert (14). Da hierbei anorganisches Phosphat und HDP grösstenteils mitgefällt wurden, konnte das Gesamtphosphat der Gärproben nicht bestimmt werden. In den Gärproben mit getrockneter Brauereihefe und Toluol wurden deswegen ein Teil der Proben mit 5%iger Trichloressigsäure enteiweisst.

Bei den Versuchen mit der unvollständig permeablen getrockneten Bäckerhefe wurde die abzentrifugierte Hefe wie diejenige der Nullproben dreimal während einer Stunde mit je 10 ml 7%iger Überchlorsäure extrahiert. Die Hefefiltrate wurden mit 7%iger kalter Überchlorsäure enteiweisst und dann mit den Säurextrakten vereinigt. Diese Lösungen wurden zu den Analysen verwendet.

In den mit Quecksilbersulfat enteiweissten Gärproben wurden die Monophosphorsäureester mit 75 % Alkohol als Bariumsalze bei pH 8,2 ausgefällt (15). In den mit Trichloressigsäure oder Überchlorsäure extrahierten oder enteiweissten Proben wurde das Gesamtphosphat zuerst auf dieselbe Weise ausgefällt. Die Fällung wurde dann in verdünnter Salzsäure aufgelöst und anorganisches Phosphat und HDP mit

10 % Alkohol bei pH 8,2 abgeschieden (2). In den Filtraten wurden die Monophosphorsäureester bestimmt. Eine kleine Einmengung von HDP konnte hier mit der papierchromatographischen Methode oft nachgewiesen werden.

Die Phosphorsäurebestimmungen wurden nach Teorell ausgeführt (16). Säurelabiles Phosphat wurde nach 10 Min. Hydrolyse in N Salzsäure bei 100° bestimmt.

Die Mengen von THP in den Monophosphatfraktionen wurden sowohl aus dem optischen Drehungswert als auch aus der Zunahme des Reduktionswertes nach fünfstündiger Hydrolyse in N Schwefelsäure bei 100° bestimmt. Keine von diesen Methoden gibt aber einwandfreie Werte, da die übrigen Phosphorsäureester bei beiden Analysenmethoden interferieren. Mit der papierchromatographischen Methode (siehe weiter unten) konnten in den Monophosphatfraktionen aller Gärproben die folgenden Ester nachgewiesen werden: G-6-P, G-1-P, F-6-P und THP. Die spezifischen Drehungen dieser Ester sind: +34,2° (17), +120° (18), +2,5° (19), +156,8° (2).[1] In den Gärproben mit Bäckerhefepräparaten wurde auch F-1-P gefunden. Die spezifische Drehung dieses Esters ist −52,5° (20). Wenn der direkte Reduktionswert der Monophosphatfraktionen im Vergleich mit der Gesamtmenge des organischen Phosphates niedrig war, konnte die Menge von THP annähernd aus der optischen Aktivität bestimmt werden. Die Ester, die wegen hoher Drehungswerte bei diesen Bestimmungen am meisten interferieren, sind G-1-P und F-1-P. Nach den papierchromatographischen Analysen zu beurteilen kamen sie aber nur in kleinen Mengen vor. Das säurelabile Phosphat gibt auch ein Mass der Mengen von G-1-P und F-1-P an, denn nach 10 Min. in N Salzsäure bei 100° sind 100 % von G-1-P und 77 % von F-1-P hydrolysiert worden, von G-6-P und F-6-P aber nur 0,5 bzw. 8 % (13).

Robison und Morgan fanden, dass THP bei der Hydrolyse in N Schwefelsäure bei 100° hauptsächlich in Glucose und G-6-P zerlegt wird (2). Die Bindung zwischen den Glucosemolekülen wird erheblich schneller als diejenige zwischen Glucose und Phosphorsäure gelöst. Aus der Hydrolysenkurve geht hervor, dass nach 5 Stunden etwa 70 % der Glucosebindungen aber nur 6 % der Bindungen zwischen Glucose und Phosphorsäure gelöst sind. In der vorliegenden Untersuchung ist bei den Bestimmungen von THP damit gerechnet worden, dass die Hydrolysenprodukte des Esters aus gleichen Teilen Glucose und G-6-P bestehen und dass der Reduktionswert von G-6-P 81 % von demjenigen der Glucose entspricht (13). Der Fehler des Reduktionswertes, der durch die Vernachlässigung der Hydrolyse der aus THP entstandenen G-6-P bewirkt wird, beträgt weniger als 1 %. Der Hydrolysegrad von THP wurde in jedem Versuch durch die Hydrolyse einer Standardprobe von THP festgestellt, die gleichzeitig mit den Gärproben hydrolysiert wurde.

Der direkte Reduktionswert der verschiedenen Hexosemonophosphorsäureester wird, je nach der Art des Esters, während der Säurehydrolyse verändert. G-1-P wird wie THP erst nach der Hydrolyse reduzierend. Der Reduktionswert von G-6-P nimmt unbedeutend zu während die Reduktionswerte von F-6-P und F-1-P etwas abnehmen, was mit einem teilweisen Abbau der Fructose zusammenhängt. Die Veränderungen sind an und für sich nicht beträchtlich, wenn aber die Mengen dieser Ester im Verhältnis zu THP bedeutend sind, können sie auf die Bestimmungen von THP wesentlich einwirken.

Die Trehalose wurde wie in der früheren Untersuchung aus dem optischen Drehungswert und aus der Zunahme des Reduktionswertes nach fünfstündiger Hydrolyse in N Schwefelsäure bei 100° bestimmt. Da die Ausfällung des Bariumsalzes nie ganz

[1] Bezüglich der Umrechnung α_{5461} in α_D siehe weiter unten.

vollständig war, wurde bei den Bestimmungen von Trehalose auch kleine Mengen von THP mitbestimmt. Diese Mengen konnten jedoch mit Hilfe der papierchromatographischen Analysen approximativ geschätzt werden.

Phosphorsäureester und Zucker wurden in den erhaltenen Ester- und Trehalosefraktionen mittels Papierchromatographie untersucht. Mit der absteigenden Methode wurden die Phosphorsäureester bei 20° sowohl mit alkalischem als auch mit saurem Lösungsmittel getrennt (n-Propanol-konz. Ammoniak-Wasser (60:30:10), tert. Butanol-Wasser-Pikrinsäure (40:10:2 g)) (8). Vor dem Chromatographieren wurden die Proben mit Kationenaustauscher behandelt (21). Als Papier wurde durchgehend Whatman 54 benutzt. Die Phosphorsäureester wurden mit dem Molybdatreagens von Hanes und Isherwood besprüht (8). Das Papier wurde während 2 Min. bei 70°–75° getrocknet und danach mit UV-Licht bestrahlt (21).

Zur Trennung der Di- und Monosaccharide wurde ein einphasiges Lösungsmittel benutzt, dass aus Äthylacetat-Eisessig-Wasser (60:17:17,5) bestand (22). Reduzierende und nicht reduzierende Zucker wurden mit Silbernitrat in Aceton und alkoholischer Natronlauge entwickelt (23).

Um die aus Gärproben isolierte THP zu identifizieren wurde die Spaltung durch alkalische Phosphatase untersucht. Ein Trockenpräparat des Enzyms wurde aus Metatarsalia der Vorderbeine eines frischgeschlachteten Ochsen hergestellt, wobei die von Martland und Robison angegebene Methode zur Anwendung kam (24). Die von Fleisch und Sehnen befreiten und zerkleinerten Knochenstücke wurden mit der fünffachen Gewichtsmenge Wasser und einigen ml Chloroform versetzt und täglich während 8 Tage gut durchgeschüttelt. Dann wurde durch ein grobes Filter filtriert und die Phosphatasen (Mono- und Diesterasen) mit 2 Tl. Methanol und 3 Tl. Äther ausgefällt, im Kühlraum abzentrifugiert und im Exsiccator über Phosphorpentoxyd getrocknet. Aus 2 Liter Extrakt wurde 1,49 g Trockenpulver erhalten. Fast die ganze Phosphataseaktivität konnte aus dem Trockenpulver mit Wasser ausgelöst werden.

Bei der Bestimmung der Phosphataseaktivität war die Zusammensetzung der Reaktionsmischung die folgende: 1 ml 0,025 M Natrium-β-glycerophosphat, 0,4 ml 0,05 M Boratpuffer nach Clark und Lubs (pH = 8,5), 0,1 ml 0,1 M Magnesiumsulfat, 1,0 ml Enzymlösung (4,5 mg Trockengewicht/ml). Die Aktivitätsbestimmungen wurden bei 38° ausgeführt. Proben von je 1 ml wurden für die Bestimmung des freigesetzten Phosphates herausgenommen. Nach 75 Min. waren 52,7 % des Esters hydrolysiert worden.

Versuchsergebnisse

Getrocknete Brauereihefe und Toluol

Die Verteilung des organischen Phosphates und der optisch aktiven Substanzen auf die verschiedenen Fraktionen ergibt sich aus der Tabelle 1. Die Tabelle 2 zeigt die direkten und die nach der Säurehydrolyse erhaltenen Reduktionswerte derselben Proben.

In den Gärproben kamen 54 % des organischen Phosphates als Diphosphat und 46 % als Monophosphat vor. Aus den direkten Reduktionswerten der Monophosphorsäureester ergab sich, dass nur etwa 5 % der Ester reduzierend wirkten. 9 % des Monophosphates waren säurelabil. Die Hauptmenge der Monophosphorsäureester war demnach nicht reduzierend und nicht säurelabil.

Tab. 1. THP und Trehalose in Gärproben mit getrockneter Brauereihefe und Toluol.

110 μMol anorganisches Phosphat und 14 μMol organisches Phosphat (als Hexosediphosphat) wurden pro Gärprobe zugesetzt. THP und Trehalose wurden aus der optischen Aktivität berechnet und die Analysenwerte in μMol pro Gärprobe angegeben.

			Filtrat nach der Enteiweissung		Diphosphatfraktion		Monophosphatfraktion					Trehalosefraktio	
Nr.	Gärzeit in Min.	Vergorene Glucose	Anorg. Phosphat	Org. Phosphat	Anorg. Phosphat	Org. Phosphat	Anorg. Phosphat	Org. Phosphat	Säurelabiles Phosphat	α_D in 2 dm-Rohr	THP	Org. Phosphat	α_D in 2 dm-Rohr
1.[1]				11,7				1,3		0,00°		9,4	+0,43°
2.[1]			45,8	55,5	45,8	39,0		10,8		−0,02°		2,9	+0,97°
3.[1]										+0,05°		0,3	+0,90°
4.	205	305	0,0	81,3			0,0	70,9	6,0	+3,41°	51,5	3,3	+1,97°
5.	205	305	0,0	80,3			0,0	65,8	6,2	+3,55°	53,6	11,6	+1,99°
6.	205	305	34,6	179,4	32,4	88,2							

[1] 1. mit Wasser, 2. mit 5 %iger Trichloressigsäure, 3. mit 7 %iger Überchlorsäure extrahierter Hefe.

Die Monophosphatfraktionen der Nullproben und der Gärproben wurden mit dem alkalischen und dem saurem Lösungsmittel chromatographiert. In den Gärproben wurden im alkalischen Lösungsmittel G-6-P, F-6-P und G-1-P getrennt, im sauren Lösungsmittel THP, G-6-P und F-6-P. Die R_f-Werte der letztgenannten Ester waren 0,18, 0,29 und 0,39. In den Nullproben kamen dieselben Ester mit Ausnahme von THP vor. Nach der Intensität der Flecken zu beurteilen, bestanden die Monophosphatfraktionen der Gärproben überwiegend aus THP. Wenn THP aus der optischen Aktivität und aus der Zunahme des Reduktionswertes berechnet wurde, stimmten die Werte ziemlich gut überein. Die Hauptmenge der Trehalosemonophosphorsäure musste während der Gärung neugebildet worden sein, da die Nullproben in der Monophosphatfraktion keine optische Aktivität zeigten und da THP chromatographisch nicht nachgewiesen werden konnte.

In den Trehalosefraktionen ist die Menge des organischen Phosphates der Probe 5 etwa dreimal so hoch wie diejenige der Doppelprobe 4, was wahrscheinlich mit einer unvollständigeren Ausfällung der Monophosphorsäureester der Probe 5 zusammenhing, denn die Menge des Gesamtmonophosphates ist in den beiden Proben gleich gross.

Die Trehalosebestimmungen aus der optischen Aktivität und aus der Zunahme des Reduktionswertes stimmten sowohl für die Nullproben als auch für die Gärproben gut überein. Es ergab sich, dass die im Wasserextrakt der Hefe gefundene Trehalosemenge nur etwa 45 % derjenigen in den Säureextrakten betrug. Ob der Unterschied durch unvollständige Extraktion mit Wasser oder durch einen Abbau der Trehalose während der Extraktion entstand, wurde nicht näher untersucht.

Die Trehalosefraktionen wurden sowohl im sauren als auch im alkalischen Lösungsmittel papierchromatographisch untersucht. Im sauren Lösungsmittel konnten in den Proben 2 und 4 nur THP nachgewiesen werden, in der Probe 5 kamen auch G-6-P und F-6-P vor. Im alkalischen Lösungsmittel wurde zudem in allen Proben ein

Tab. 2. THP und Trehalose in Gärproben mit getrockneter Brauereihefe und Toluol.

Die Reduktionswerte wurden als Glucose berechnet und THP und Trehalose aus der Zunahme des Reduktionswertes während der Säurehydrolyse bestimmt. Da THP dabei in Glucose und G-6-P zerlegt wird, wurde eine Korrektion wegen des zu niedrigen Reduktionswertes von G-6-P (81 % von demjenigen der Glucose) eingeführt.

	Monophosphatfraktion				Trehalosefraktion			
	Reduzierende Substanzen (μMol Glucose)				Reduzierende Substanzen (μMol Glucose)			
Nr.	vor Hydrolyse	nach Hydrolyse	Hydrolysefaktor von THP	THP in μMol	vor Hydrolyse	nach Hydrolyse	Hydrolysefaktor der Trehalose	Trehalose in μMol
1.					0,8	12,5	1,09	6,4
2.					2,8	30,5	1,09	15,1
4.1	3,3	72,3	1,43	54,5	1,1	52,2	1,16	29,6
5.1	3,9	75,1	1,43	56,3	3,9	57,9	1,16	31,3

unbekannter Ester beobachtet. Nach der Intensität der Flecken zu beurteilen, war die Menge dieses Esters bedeutend niedriger als diejenige von THP. Bei den oben genannten Analysenmethoden der Trehalose wurde auch THP mitbestimmt. Da das organische Phosphat der Proben 2 und 4, nach den papierchromatographischen Analysen zu beurteilen, hauptsächlich aus THP bestand, können die Trehalosewerte dieser Proben wegen THP korrigiert werden. Nach der Korrektion ist der Trehalosegehalt, aus der optischen Aktivität berechnet, 11,5 bzw. 25,9 μMol pro Gärprobe. Die Trehalose nahm während der Gärung um etwa 14 μMol zu, die 5 % der zugesetzten Glucose entsprachen. Gleichzeitig hatten sich 52 μMol THP gebildet, die mit 18,7 % der zugesetzten Glucose equivalent waren.

Isolierung der Trehalosemonophosphorsäure

Aus 12 Gärproben von je 20 ml mit getrockneter Brauereihefe und Toluol konnte fast reine Trehalosemonophosphorsäure isoliert werden. Nach dreistündiger Gärung, wobei 50 % der zugesetzten Glucose vergoren worden waren, wurde die Gärung unterbrochen, die Hefe abzentrifugiert und die Proben mit 5%iger kalter Trichloressigsäure enteiweisst. Aus dem Filtrat, das 78 % der ursprünglichen Reaktionsmischung enthielt, wurden Di- und Monophosphorsäureester wie üblich gefällt. Etwa ein Drittel der Monophosphorsäurefällung, auf dem Phosphorwert bezogen, war im Wasser unlöslich und bestand vorwiegend aus HDP. Der wasserlösliche Teil der Fällung wurde mit 30 und 80 % Alkohol fraktioniert. Tabelle 3 zeigt die Phosphorsäuremengen, die direkten Reduktionswerte und die optische Aktivität der Fraktionen. Aus der optischen Aktivität liess sich die Menge von THP zu 61,0 μMol pro Gärprobe von 2 ml berechnen. Die mit 30 % Alkohol gefällte Fraktion enthielt etwa 60 % der Gesamtmenge von THP. Diese Fraktion wurde zur Isolierung des Brucinsalzes von THP verwendet. Das Brucinsalz wurde nach Veibel (3) mit 60 % Aceton gefällt. Nach dem Trocknen im Vakuumexsiccator über konz. Schwefelsäure betrug das Gewicht des Salzes 3,13 g $[\alpha]_D = +26{,}1°$ (c = 1,57 im Wasser). Robison und Morgan fanden $[\alpha]_{5461} + 31{,}3°$ (2). Nach Bolzmanns Formel (25) lässt sich der

Tab. 3. Isolierung der Trehalosemonophosphorsäure aus Gärproben mit getrockneter Brauereihefe und Toluol.

THP und Trehalose aus der optischen Aktivität bestimmt. Analysenwerte in μMol pro Gärprobe berechnet.

Fraktion	Anorg. Phosphat	Org. Phosphat	Direkt reduzierende Substanz	α_D in 2 dm-Rohr	THP	Isolierte THP	Trehalose
Proteinfiltrat	34,1	179,0					
Diphosphat	29,7	69,2		+0,10°			
In Wasser schwerlösliches Gemisch von Di- und Monophosphat[1]	2,8	28,2	6,5	+0,72°	10,9		
Mit 30 %igem Alkohol gefälltes Monophosphat		49,1	2,3	+2,42°	36,6	20,2	
Mit 80 %igem Alkohol gefälltes Monophosphat		15,8	0,6	+0,86°	13,0		
Esterfiltrat		9,8		+1,82°			27,0

[1] Vergleiche hier Robison und Morgan (26).

Faktor für die Umrechnung von α_{5461} in α_D zu 1,18 berechnen. Unter Umrechnung mit diesem Faktor ergibt sich +26,8°. Das Brucinsalz wurde in das Bariumsalz umgewandelt. Nach dem Trocknen über Phosphorpentoxyd bei 100° und 1 mm Hg war das Gewicht 1,09 g und der Phosphorgehalt 5,39 %, was dem berechneten Phosphorwert des Bariumsalzes von THP mit 1 Mol Kristallwasser entspricht. Auf den Phosphorwert bezogen war $[\alpha]_D$ des freien Esters +154,8° (c = 1,82). Nach Robison und Morgan ist $[\alpha]_{5461} = +185°$ (c = 2,4). Für $[\alpha]_D$ ergibt sich denn, nach Bolzmanns Formel umgerechnet, +156,8°. Die Ausbeute an Bariumsalz betrug 55 % der aus der optischen Aktivität berechneten Totalmenge von THP in dieser Fraktion.

THP wurde von Robison und Morgan (2) mit Knochenphosphatase hydrolysiert, und aus der Reaktionsmischung konnte kristallisierte Trehalose isoliert werden. Die im experimentellen Teile beschriebene Knochenphosphatase durfte auf den hier isolierten Ester einwirken. Die Konzentration des Esters war in der Reaktionsmischung 0,01 M. Nach 75 Min. bei 38° waren 50,7 % des organischen Phosphates hydrolysiert worden. Die Reaktionsmischung wurde eingeengt und mit Kationenaustauscher (Dowex 50) behandelt. Die in dieser Weise behandelte Probe wurde gleichzeitig mit einer unbehandelten Probe in Äthylacetat-Eisessig-Wasser (22) chromatographiert. Glucose und Trehalose wurden als Vergleichssubstanzen verwendet. Nur die mit Phosphatase vorbehandelte Probe enthielt einen Stoff mit dem R_f-Wert der Trehalose.

Die Trehalosemonophosphorsäure wurde sowohl im alkalischen als auch im sauren Lösungsmittel chromatographiert. Eine kleine Beimengung eines unbekannten Esters konnte nachgewiesen werden. Der direkte Reduktionswert der isolierten Trehalosemonophosphorsäure betrug (als Glucose berechnet) 1,9 % der aus dem Phosphorgehalte berechneten Menge des Esters und deutete auf eine Beimengung eines reduzierenden Esters hin.

Getrocknete Bäckerhefe und Toluol

Die zu den Gärversuchen mit getrockneter Bäckerhefe und Toluol gehörigen Analysenwerte sind in den Tabellen 4 und 5 wiedergegeben.

In einem Vorversuch mit diesem Hefepräparat, in welchem die Proben nach verschiedenen Gärzeiten herausgenommen wurden, zeigte sich, dass sich während der Gärung eine gewisse Menge von THP bildete, die am Ende der Glucosegärung wieder schnell abgebaut wurde. Die Proben 3 und 4 (Tab. 4 und 5) wurden deswegen herausgenommen, wenn etwa ein Drittel der Glucose vergoren worden war, während die Gärung der Proben 5 und 6 wie im Versuch mit Brauereihefe am Ende der Glucosegärung abgebrochen wurde. Die Gärung der Probe 6 verlief etwas schneller als diejenige der Probe 5. Da die Proben nach derselben Gärzeit herausgenommen wurden, war die Gärung der Probe 6 etwa 15 Min. vor der Herausnahme der Probe schon beendet, was sich in einem grösseren Abbau des organischen Phosphates dieser Probe zu erkennen liess. Der kleine Unterschied der Gärgeschwindigkeiten hatte wahrscheinlich seinen Grund darin, dass die Hefe der Probe 6 vor der Gärung besser suspendiert wurde als diejenige der Probe 5.

In den Gärproben war das Gesamtphosphat der Eiweissfiltrate um etwa 17 % höher als die zugesetzte und die in den Nullproben gefundene Menge. Während der Gärung wurde somit mehr organisches Phosphat aus der Hefe freigemacht, als was mit Säure direkt extrahiert werden konnte. In den Fraktionen wurden etwa 90 % des anorganischen Phosphates und 86–87 % des organischen Phosphates der Eiweissfiltrate wiedergefunden. In den Proben 3 und 4 waren die Mengen von Diphosphat und Monophosphat etwa gleich gross. In den bei beendeter Gärung herausgenommenen Proben war die Menge des Diphosphates dagegen prozentuell höher und betrug 63 bzw. 65,8 %. Die Menge des organischen Phosphates war in diesen Proben stark vermindert. Die Abnahme hatte vorwiegend das Monophosphat getroffen.

Wie die Vorproben dieser Versuchsreihe schon angedeutet hatten, konnte in der Monophosphatfraktion der Proben 3 und 4 eine deutliche optische Aktivität nachgewiesen werden. In der Probe 5 war sie im Vergleich mit den Proben 3 und 4 bis auf ein Drittel abgesunken und in der Probe 6 war die positive Drehung in eine schwach negative übergegangen. In den Nullproben war die optische Aktivität auch schwach negativ. Die in der Hefe vorhandene Menge von THP muss deswegen äusserst gering sein. Wenn THP in den Proben 3 und 4 aus der optischen Aktivität und aus der Zunahme des Reduktionswertes berechnet wird, werden gut übereinstimmende Werte erhalten. In den Proben 5 und 6, wo wenig THP vorliegt, kann wegen der Einwirkung von HMP eine Übereinstimmung der Analysenwerte nicht erwartet werden.

Die papierchromatographische Trennung der Monophosphatfraktionen im alkalischen und im sauren Lösungsmittel sind in den Figuren 1 und 2 wiedergegeben. Im alkalischen Lösungsmittel konnten von der Startlinie aus die folgenden Ester nachgewiesen werden: F-1,6-P, G-6-P, F-6-P und G-1-P. Die Trennung der Esterphosphate war im sauren Lösungsmittel weniger scharf als im alkalischen, in der Probe 6 war sie jedoch am deutlichsten. Von der Startlinie aus war die Reihe der Ester die folgende: THP, G-6-P mit G-1-P, F-1-P, F-6-P mit F-1,6-P, Spuren von anorganischem Phosphat. Aus der Figur 2 geht hervor, dass THP in allen Gärproben vorkam, auch in der Probe 6, wo die optische Aktivität schwach negativ war. In den Nullproben wurden nur Spuren von diesem Ester gefunden.

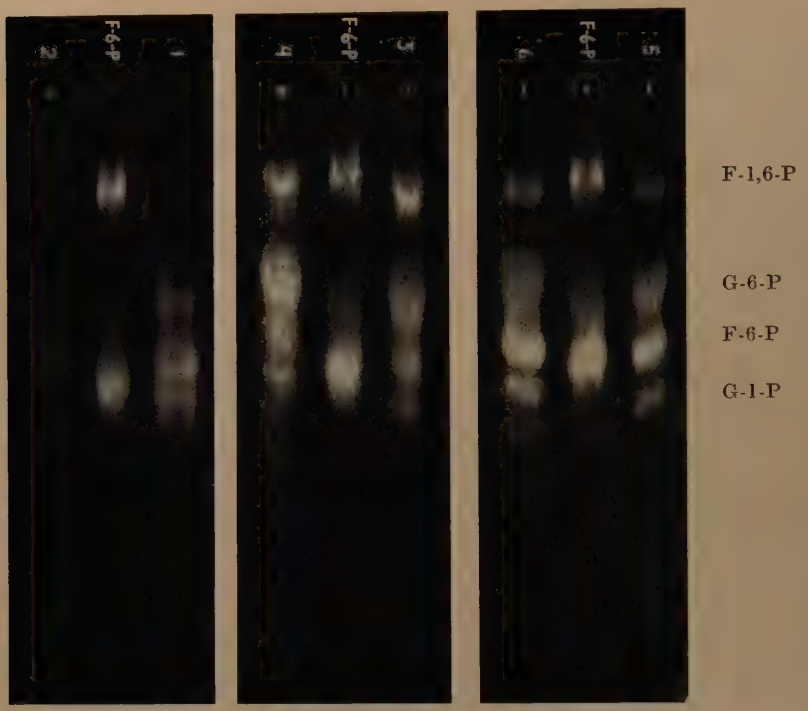

Fig. 1. Monophosphatfraktionen der Nullproben und Gärproben mit getrockneter Bäckerhefe und Toluol. Lösungsmittel: n. Propanol: konz. Ammoniak; 15–17 Stunden. Die analysierte totale Estermenge: 20–30 μg. Als Vergleichssubstanz ist wegen Raumersparnis nur F-6-P (Boeringer, Mannheim) mitgenommen; etwa 15 μg. Die übrigen Vergleichssubstanzen wurden von der Firma Sigma, St. Louis, erhalten. Die Nummer der Proben wie in den Tabellen 4 und 5.

Bei den Berechnungen der Trehalose aus der optischen Aktivität, mussten für die Proben 3 und 4 eine Korrektion wegen Gegenwart freier Glucose angebracht werden. Die Glucosemengen wurden aus den direkten Reduktionswerten der Tabelle 5 erhalten ($[\alpha]_D$ der Glucose $+52{,}5°$) (28).

Das organische Phosphat der Trehalosefraktionen bestand fast ausschliesslich aus THP, was mit der papierchromatographischen Methode nachgewiesen wurde. Die Trehalosewerte konnten deshalb wegen Gegenwart von THP korrigiert werden. In den Gärproben bestanden 3–4 % der als Trehalose bestimmten Substanze aus THP. In den Nullproben betrug der THP-Wert etwa 2 % desjenigen der Trehalose.

Aus der Tabelle 4 ergibt sich, dass in den Proben 3 und 4 etwa 9 % der zugesetzten Glucose in THP und 14,5 % in Trehalose umgewandelt worden sind. In den Proben 5 und 6 ist der Trehalosewert bis auf 29,5 % gestiegen. Die THP-Werte sind hier ziemlich ungenau und betrugen höchstens etwa 3 % der zugesetzten Glucose. Die Synthese der Trehalose erscheint auf Kosten des Trehalosemonophosphates vorzugehen. Werden die Werte des anorganischen Phosphates und diejenigen der Trehalose der Probe 5 mit dem Mittelwert der Proben 3 und 4 verglichen, wird gefunden, dass das anorganische Phosphat um 36,4 μMol und die Trehalose um 41,3 μMol zugenommen haben. Es wird vermutet, dass, so lange freie Glucose anwesend ist,

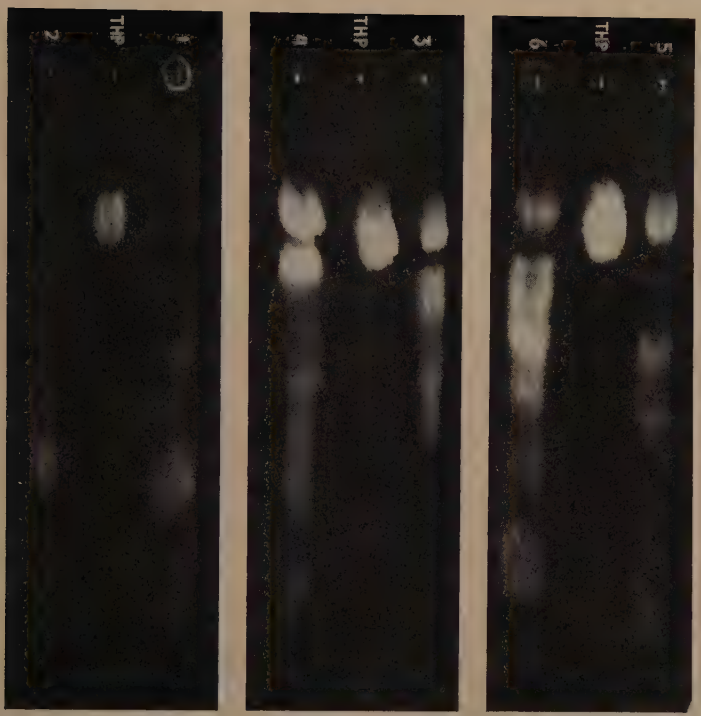

Fig. 2. Monophosphatfraktionen der Nullproben und Gärproben mit getrockneter Bäckerhefe und Toluol. Lösungsmittel: tert. Butanol: Pikrinsäure; 24 Stunden. Die analysierte totale Estermenge: 20–30 μg. Vergleichssubstanz: THP (aus Gärmischung mit getrockneter Brauereihefe und Toluol isoliert); 15 μg. F-1-P wurde nach Pogell (27) hergestellt. Die Nummer der Proben wie in den Tabellen 4 und 5.

das freigesetzte anorganische Phosphat hauptsächlich aus dem Trehalosemonophosphat herrührt.

Der Unterschied der Mengen von THP und Trehalose, die nach beendeter Gärung mit Brauerei- bzw. Bäckerhefe nachgewiesen werden konnten, war auffallend gross und wird in der Diskussion näher besprochen.

Acetongetrocknete und gewaschene Bäckerhefe ohne und mit Toluol

Die oben beschriebenen mit getrockneter Bäckerhefe ausgeführten Versuche machten das Vorkommen von THP auch bei der Acetonhefegärung sehr wahrscheinlich. In den vorher mit Acetonhefe ausgeführten Untersuchungen (1) war jedoch die optische Aktivität aller Proben in der Monophosphatfraktion sehr niedrig, auch in denjenigen Proben, die in Gegenwart freier Glucose herausgenommen wurden. Die Möglichkeit, dass die Menge von THP in Gegenwart von Toluol erhöht werden konnte, war aber nicht auszuschliessen, da die Adenylpyrophosphatase, die durch das Trockenverfahren teilweise inaktiviert wird (11), weiter durch Toluol gehemmt werden konnte (11). In einigen mit acetongetrockneter und gewaschener Bäckerhefe neu ausgeführten Gärproben wurde deswegen die Einwirkung von Toluol auf die Synthese von THP und Trehalose nachgeprüft.

Tab. 4. THP und Trehalose in Gärproben mit getrockneter Bäckerhefe und Toluol.

100 μMol anorganisches Phosphat und 14 μMol organisches Phosphat (als Hexosediphosphat) wurden pro Gärprobe zugesetzt. THP und Trehalose wurden aus der optischen Aktivität berechnet und die Analysenwerte in μMol pro Gärprobe angegeben.

			Filtrat nach der Enteiweissung		Diphosphatfraktion			Monophosphatfraktion					Trehalosefraktio		
Nr.	Gärzeit in Min.	Vergorene Glucose	Anorg. Phosphat	Org. Phosphat	Anorg. Phosphat	Org. Phosphat	α_D in 2 dm-Rohr	Anorg. Phosphat	Org. Phosphat	Säure labiles Phosphat	α_D in 2 dm-Rohr	THP	Org. Phosphat	α_D in 2 dm-Rohr	
1.			9,9	41,7	12,3	13,0	−0,03°	0,2	13,4	2,0	−0,03°		1,6	+ 5,36°	7
2.			9,5	41,7	12,5	13,5	−0,01°	0,3	13,7	2,0	−0,05°		1,7	+ 5,40°	8
3.	145	213,9	34,2	164,5	30,8	70,0	+0,06°	1,2	65,8	9,3	+1,33°	20,1	5,9	+ 8,22°[1]	12
4.	145	210,0	31,9	164,1	28,5	73,4	+0,09°	0,9	64,2	7,2	+1,49°	22,5	4,5	+ 8,44°[1]	12
5.	285	308,9	72,6	122,6	65,0	68,5	+0,10°	2,1	33,9	6,9	+0,41°	6,2	5,4	+11,13°	16
6.	285	311,1	91,6	99,4	80,9	56,5	+0,09°	2,0	23,1	4,7	−0,08°		6,2	+11,23°	16

[1] Wegen Gegenwart freier Glucose korrigiert.

Tabelle 6 zeigt die Analysenwerte der Acetonhefegärungen. Probe 1 und 2 sind die Nullproben der Hefe und des Hefekochsaftes. Von den vier Gärproben wurden zwei mit frisch getrockneter Hefe vergoren, eine ohne und eine mit Toluol (Probe 3 und 5). Zwei Proben wurden mit demselben Hefepräparat, ohne und mit Toluol, 3 Monate später ausgeführt (Probe 4 and 6). Die Hefe wurde in der Zwischenzeit im Kühlschrank aufbewahrt.

Tab. 5. THP und Trehalose in Gärproben mit getrockneter Bäckerhefe und Toluol.

THP und Trehalose wurden wie in der Tabelle 2 aus der Zunahme des Reduktionswertes während der Säurehydrolyse bestimmt.

	Monophosphatfraktion				Trehalosefraktion			
	Reduzierende Substanzen (μMol Glucose)				Reduzierende Substanzen (μMol Glucose)			
Nr.	vor Hydrolyse	nach Hydrolyse	Hydrolysefaktor von THP	THP in μMol	vor Hydrolyse	nach Hydrolyse	Hydrolysefaktor der Trehalose	Trehalose in μMol
1.	0,0				1,8	135,1	1,14	76,0
2.	0,0				1,9	134,6	1,14	75,6
3.	20,3	51,9	1,17	20,4	119,8	334,1	1,14	122,2
4.	22,6	58,2	1,17	23,0	131,7	348,5	1,14	123,6
5.	12,5	27,7	1,17	9,8	8,5	296,0	1,14	163,9
6.	8,9	16,4	1,17	4,8	9,5	299,5	1,14	165,3

Tab. 6. Trehalose in Gärproben mit acetongetrockneter und gewaschener Bäckerhefe ohne und mit Toluol.

Trehalose aus der optischen Aktivität und aus der Zunahme des Reduktionswertes während der Säurehydrolyse bestimmt. Analysenwerte in μMol pro Gärprobe berechnet.

Gärzeit in Min.	Mit Acetonhefe vergorene Glucose	Mit frischer Bäckerhefe vergorene Glucose	Monophosphatfraktion			Trehalosefraktion					
			Org. Phosphat	Direkt reduz. Subst. (als Glucose)	α_D in 2 dm-Rohr	Org. Phosphat	α_D in 2 dm-Rohr	Trehalose	Reduzierende Substanzen (als Glucose) vor Hydrolyse	Reduzierende Substanzen (als Glucose) nach Hydrolyse	Hydrolysefaktor der Trehalose	Trehalose
			11,0		−0,03°	0,3	0.00°					
			1,6		+0,04°	3,6	+0,80°	11,9	0,0	23,3	1,09	12,7
155	312	0,3	42,3	11,1	+0,07°	8,4	+4,74°	70,3	1,7	132,0	1,11	72,3
195	311	34,0	43,6	13,9	+0,00°	17,7	+4,38°	65,0	1,7	125,0	1,11	68,4
225	312	38,0	51,3	15,5	+0,11°	5,5	+5,04°	74,8	1,7	126,0	1,24	77,1
250	312	23,4	52,3	21,1	0,00°	16,4	+5,03°	74,7	1,7	139,2	1,11	76,3

[1] 1. in acetongetrockneter und gewaschener Bäckerhefe 2. im Hefekochsaft.

Die Acetonhefegärungen wurden in allen Proben abgebrochen, wenn 56 % der zugesetzten Glucose vergoren worden waren. Die Gärgeschwindigkeit wurde sowohl mit der gealterten Hefe als auch in Gegenwart von Toluol beträchtlich herabgesetzt. Wenn die Acetonhefegärungen abgebrochen wurden, war die freie Glucose nur in der Probe 3 völlig verbraucht, was aus den mit frischer Bäckerhefe ausgeführten Nachgärungen hervorging.

Aus der Tabelle geht hervor, dass die Gesamtmenge der Monophosphorsäureester in den Toluolproben etwa 12 % höher war als in den Proben ohne Toluol. Mehr Monophosphat wurde auch in den mit gealterter Hefe vergorenen Proben gefunden als in denen mit frisch hergestellter Acetonhefe. Nur in den Monophosphatfraktionen der letztgenannten konnte eine äusserst geringe optische Aktivität beobachtet werden. Eine erhöhte Menge von THP wurde in den Toluolproben nicht gefunden.

Die chromatographische Untersuchung der Trehalosefraktionen zeigte, dass das organische Phosphat hauptsächlich aus THP bestand. Zwei unbekannte Phosphorsäureverbindungen kamen jedoch zum Vorschein. In den Proben 4 und 6 war die Ausfällung des Hexosemonophosphates ausserdem unvollständig. Bei den Trehalosebestimmungen konnte deswegen keine sichere Korrektion wegen THP angebracht werden. Soweit man aus den einfachen Proben dieser Untersuchung beurteilen darf, wurde die Synthese der Trehalose in Gegenwart von Toluol erhöht. Der Unterschied der Proben 3 und 5 ist 7,5 %. Wahrscheinlich würde er noch etwas grösser sein, wenn die Gärung der Toluolproben bis zur vollständiger Erschöpfung der freien Glucose fortgesetzt worden wäre.

Die Untersuchung über die Zusammensetzung der Phosphorsäureesterfraktionen dieser Gärmischungen wird fortgesetzt. Versuche sind im Gange, die während der Glucosegärung eintretenden Verschiebungen in den Mengenverhältnissen der verschiedenen Phosphorsäureester quantitativ zu verfolgen.

Tab. 7. THP und Trehalose in Gärproben mit Extrakt aus autolysierter Bäckerhefe.

THP und Trehalose aus der optischen Aktivität bestimmt. Analysenwerte in μMol pro Gärprobe berechnet.

Nr.	Gärzeit in Min.	Vergorene Glucose	Monophosphatfraktion			Trehalosefraktion		
			Org. Phosphat	a_D in 2 dm-Rohr	THP	Org. Phosphat	a_D in 2 dm-Rohr	Trehalose
1.			0,0			5,7	+2,49°	36.9
2.	250	259	20,0	+0,40°	6,0	2,8	+7,65°	113,5
3.	250	265	34,9	+0,48°	7,3	3,4	+7,72°	114,6
4.			0,0			3,1	+3,89°	57,7
5.	250	218	51,7	+1,56°	23,6	3,3	+6,71°	99,6
6.	250	218	62,6	+1,71°	25,8	3,4	+7,58°	112,5

Extrakte aus autolysierter Bäckerhefe

Elander und Myrbäck (29) haben gezeigt, dass mit Mazerationssaft aus Brauereihefe etwa 20 % der zugesetzten Glucose in Trehalose umgewandelt werden können. Vergleicht man diese Menge mit derjenigen, die in der vorliegenden Untersuchung mit getrockneter Brauereihefe und Toluol gefunden wurde, findet man, dass die Trehalosesynthese des Mazerationssaftes, auf verbrauchte Glucose berechnet, etwa dreimal so gross war wie diejenige der Trockenhefe, von welcher statt Trehalose vorwiegend THP synthetisiert wurde. Die Menge von THP wurde in den Versuchen mit Mazerationssaft nicht bestimmt. Mehrere Beobachtungen zeigten aber, dass sie nicht annähernd so gross sein konnte wie bei den Trockenhefegärungen; 85 % der aus der optischen Aktivität berechneten Trehalosemenge konnten in kristallisierter Form isoliert werden; die optische Aktivität wurde durch die Ausfällung der Monophosphate sehr wenig beeinflusst und der P-Gehalt des Filtrates nach Fällung des Monophosphates war unbedeutend (nicht publiziert). Nach diesen Versuchen zu beurteilen, erfolgt die Dephosphorylierung von THP durch den Saft viel schneller als durch die Trockenhefe.

Im Anschluss an die Untersuchung über die Synthese von THP und Trehalose mit verschiedenen Trockenhefepräparaten wurden Versuche angestellt, das trehalosesynthetisierende Fermentsystem aus der Bäckerhefe in Lösung zu bringen. Diesbezügliche Versuche mit Extrakten aus Bäckerhefe sind früher nicht veröffentlicht worden. Wie im experimentellen Teile erwähnt, wurden Extrakte aus zwei Bäckerhefen, die bei verschiedenen Temperaturen autolysiert worden waren, hergestellt. Die Analysenwerte gehen aus den Tabellen 7 und 8 hervor. Für die Proben 1–3 wurde Saft A und für die Proben 4–6 Saft B verwendet. Die Gärversuche waren nach 250 Min. beendigt. Die direkten Reduktionswerte der Trehalosefraktionen zeigen, dass zu dieser Zeit freie Glucose fast ganz verbraucht worden war. Mit Saft A wurde mehr Glucose vergoren als mit Saft B.

Die Analysenwerte zeigen, dass sich das trehalosebildende Enzymsystem aus der autolysierten Bäckerhefe vorzüglich extrahieren lässt und dass es im Extrakte ebenso aktiv sein kann wie in der Trockenhefe. Mit Extrakt A wurde 28 % der zugesetzten Glucose in Trehalose umgewandelt, dieselbe Menge, die in den Gärproben mit getrockneter Bäckerhefe und Toluol gefunden wurde (Tab. 4). In den Extraktproben

Tab. 8. THP und Trehalose in Gärproben mit Extrakt aus autolysierter Bäckerhefe.

THP und Trehalose wurden wie in Tab. 2 aus der Zunahme des Reduktionswertes während der Säurehydrolyse bestimmt.

	Monophosphatfraktion				Trehalosefraktion			
	Reduzierende Substanzen (μMol Glucose)				Reduzierende Substanzen (μMol Glucose)			
Nr.	vor Hydrolyse	nach Hydrolyse	Hydrolysefaktor von THP	THP in μMol	vor Hydrolyse	nach Hydrolyse	Hydrolysefaktor der Trehalose	Trehalose in μMol
1.					1,4	68,7	1,15	38,7
2.	4,5	11,8	1,40	5,6	2,5	203,0	1,15	115,3
3.	5,7	14,2	1,40	6,6	2,8	202,5	1,15	114,8
4.					1,5	103,1	1,15	58,4
5.	12,1	42,2	1,40	23,3	5,2	174,5	1,15	97,4
6.	13,3	48,7	1,40	27,4	6,7	195,0	1,15	108,3

wurde etwas THP in der Monophosphatfraktion wiedergefunden. Der aus der optischen Aktivität berechnete Wert entsprach jedoch nur 2,4 % der zugesetzten Glucose. Dieser Wert muss aber nach dem oben über die Gärungen mit getrockneter Bäckerhefe Gesagten als ziemlich unsicher angesehen werden.

Das Verhältnis THP/Trehalose war in den Gärversuchen mit Extrakt B mehr nach THP verschoben. Während die Menge der Trehalose nur 15 bzw. 19,7 % der zugesetzten Glucose betrug[1], war diejenige von der ausgefällten THP bis auf 8,5 % bzw. 9,3 % erhöht. Trotzdem die Menge von HMP in diesen Versuchen erheblich höher war als in denen mit Saft A, war die Totalmenge neugebildeter THP und Trehalose etwas niedriger. Die erhöhte Ausbeute an sowohl THP als auch HMP in den Versuchen mit Extrakt B muss dadurch entstanden sein, dass in dieser Hefe gewisse Phosphatasen zufolge der höheren Autolysetemperatur weitgehender beschädigt worden waren als die der Hefe A.

Diskussion

Die möglichen Ursachen der erhöhten Trehalosesynthese mit acetongetrockneter Bäckerhefe sind in einer früheren Arbeit diskutiert worden (1). Mit getrockneter Brauerei- bzw. Bäckerhefe wird zugesetzte Glucose schnell und fast vollständig vergoren (9, 12). In Gegenwart von gewissen organischen Stoffen wie Toluol, Oktylalkohol oder Phenylurethan werden Phosphorsäureester angehäuft, was eine unvollständige Vergärung der Glucose mit sich bringt (11). Meyerhof fand, dass die Anhäufung der Phosphorsäureester auf einer Hemmung der gebundenen Adenylpyrophosphatase durch die erwähnten Stoffe beruht. Aus der vorliegenden Untersuchung geht hervor, dass in Gärversuchen mit getrockneter Brauerei- bzw. Bäckerhefe und Toluol die Anhäufung der Phosphorsäureester eine Synthese von THP und Trehalose hervorruft. In Versuchen mit acetongetrockneter Bäckerhefe wurde die Einwirkung von Toluol untersucht und eine gewisse Steigerung der Trehalosesynthese beob-

[1] Die Werte wurden wegen Gegenwart von THP nicht korrigiert.

achtet. Im Extrakt aus Bäckerhefe, wo während der Gärung eine lebhafte Umwandlung der Glucose in THP und Trehalose vorging, konnte auf Grund von Meyerhofs Ergebnissen angenommen werden, dass während der Autolyse und der Extraktion die Adenylpyrophosphatase beschädigt wurde.

Beim Vergleich der Mengen von THP und Trehalose, die am Ende der Glucosegärung mit Brauerei- bzw. Bäckerhefe vorlagen, wurde gefunden, dass mit der Brauereihefe die Gesamtausbeute an THP und Trehalose, die etwa 24 % der zugesetzten Glucose betrugen, bis auf $\frac{3}{4}$ aus THP bestanden. In der Bäckerhefe wurden nur ganz geringfügige Mengen des Esters gefunden, während etwa 30 % der zugesetzten Glucose in Trehalose umgewandelt worden waren. Die höhere Ausbeute an THP in den Brauereihefeproben kann nicht durch eine höhere Konzentration von HMP in diesen Proben erklärt werden, denn wird der Monophosphatwert wegen der Anwesenheit von THP korrigiert, wurde mehr HMP in den Bäckerhefeproben als in den Brauereihefeproben gefunden. Die Abspaltung der Phosphorsäure von THP erscheint vielmehr bei pH 6 in der getrockneten Bäckerhefe erheblich schneller als in der getrockneten Brauereihefe vorzugehen.

In Ober- und Unterhefen sind zwei verschiedene saure Phosphatasen nachgewiesen und eingehender untersucht worden. Die sog. saure Oberhefenphosphatase wurde von Albers und Albers entdeckt (30). Sie fanden eine acht- bis neunmal höhere Aktivität dieses Enzymes in Oberhefe als in Unterhefe. Das Wirkungsoptimum des gebundenen Enzymes ist breit und liegt zwischen pH 3 und 4,5. Bei pH 6 zeigt das Enzym, wenn β-Glycerophosphat das Substrat ist, etwa 50 % der optimalen Aktivität. Magnesiumionen wirken hemmend. Diese Phosphatase ist, wie auch Schäffner und Krumey gefunden haben (31), wenig spezifisch. Sie spaltet sowohl α- und β-Glycerophosphat als auch HDP, HMP, Triphosphat und Pyrophosphat. Die andere saure Phosphatase wurde von Schäffner und Bauer in Unterhefe gefunden (32). Dieses Enzym ist auf α-Glycerophosphat sehr spezifisch eingestellt und zeigt bei pH 6,4 ein scharfes Wirkungsoptimum. Tsuboi und Mitarbeiter haben später das Enzym aus Bäckerhefe in Lösung gebracht und weitgehend gereinigt (33, 34). Magnesiumionen sind für die Entfaltung der Aktivität dieses Enzymes notwendig. Ausser α-Glycerophosphat wird auch 1-Propandiolphosphat gespalten, G-1-P und G-6-P dagegen nicht. Da die phosphatatische Spaltung von THP mit diesen beiden Enzymen noch nicht untersucht ist, lässt sich ohne weitere Versuche nicht entscheiden, ob eine von diesen Phosphatasen für die Bildung von Trehalose im Innern der Hefezelle verantwortlich ist. Wie oben erwähnt fanden Cabib und Leloir in Brauereihefe eine auf THP spezifisch wirkende Phosphatase (5), die als Verunreinigung das THP-synthetisierende Enzym begleitete. Das Aktivitätsoptimum dieser Phosphatase wurde freilich nicht bestimmt. Magnesiumionen wirkten stark aktivierend, obwohl sie für die Entfaltung der Enzymaktivität nicht unvermeidlich notwendig erschienen. Von den als Substrat untersuchten Phosphorsäureverbindungen, THP, G-1-P, G-6-P, UDP und UDPG wurde nur THP von dieser Phosphatase gespalten. THP hat mit den Substraten der α-Glycerophosphatase, α-Glycerophosphat und 1-Propandiolphosphat, einige Eigenschaften gemeinsam; der Ester ist nicht reduzierend, die Phosphorsäure ist an einem primären Kohlenstoffatom gebunden. Ob die Phosphatase von Cabib und Leloir mit der α-Glycerophosphatase identisch ist, lässt sich auf Grund der vorhandenen Untersuchungen jedoch nicht entscheiden.

Nach der vorliegenden Untersuchung zu beurteilen, würde man in der Bäckerhefe eine höhere Phosphataseaktivität bezüglich THP als in der Brauereihefe erwarten können; diese Frage soll eingehender untersucht werden.

ZUSAMMENFASSUNG

Die Mengen von THP und Trehalose, die am Ende der Glucosegärung mit getrockneter Brauereihefe bzw. Bäckerhefe und Toluol vorlagen, wurden verglichen. Mit dem Brauereihefepräparat wurden von der Glucose 18,7 % in THP und 5 % in Trehalose umgewandelt. Mit der getrockneten Bäckerhefe wurden etwa 30 % der Glucose in Trehalose umgewandelt; nur geringe Mengen von THP wurden gleichzeitig beobachtet. Erheblich mehr THP wurde aber in den Proben gefunden, die vor dem Abschluss der Gärung herausgenommen wurden.

Aus der Gärmischung mit getrockneter Brauereihefe und Toluol wurde THP als Brucinsalz aus der Monophosphatfraktion isoliert. Die spezifische Drehung des freien Esters auf den Phosphorwert bezogen, war +154.8°. Nach Dephosphorylierung des Esters mit alkalischer Phosphatase wurde eine Substanz mit dem R_f-Wert der Trehalose papierchromatographisch nachgewiesen.

Mit tert. Butanol: Wasser: Pikrinsäure als Lösungsmittel wurde THP auf Papierchromatogrammen von den Hexosemonophosphorsäureestern gut getrennt. Der R_f-Wert von THP war 0,18 und derjenige von G-6-P 0,29.

Bei der Vergärung von Glucose mit acetongetrockneter und gewaschener Bäckerhefe wurde in Gegenwart von Toluol 7,5 % mehr Trehalose gefunden als ohne Toluol. THP konnte nur papierchromatographisch nachgewiesen werden.

Bäckerhefe wurde bei 19° und 28° autolysiert. Das trehalosebildende Enzymsystem konnte aus den autolysierten Hefen zusammen mit den Gärungsenzymen mit sek. Natriumphosphat extrahiert werden. Die Autolysetemperatur hatte auf das Mengenverhältnis von THP und Trehalose, die während der Gärung synthetisiert wurden, einen erheblichen Einfluss. Mehr THP und weniger Trehalose wurden mit der bei der höheren Temperatur autolysierten Hefe gebildet.

Herrn Professor Dr. K. Myrbäck danke ich für wertvolle Kritik und freundliche Beratung. Fräulein Barbro Persson sei für ihre gewissenhafte Mitarbeit bei der Durchführung der papierchromatographischen Analysen herzlich gedankt.

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A 180° mass spectrometer for analysis of organic compounds of high molecular weight

By Ragnar Ryhage

With 11 figures in the text

It was shown by O'Neal and Wier in 1951 (1) that mass spectrometric analysis of hydrocarbons of high molecular weight was possible. They used a commercial mass spectrometer that had been modified in order to increase the mass range and improve the resolution. The use of a heated intake system allowed hydrocarbons containing up to about 40 carbon atoms with molecular weights of about 600 to be successfully analysed. As it appeared possible that mass spectrometric analysis might also be applied usefully in the analysis of several types of compounds of biological interest (e.g. fatty acids) the construction of a suitable instrument was begun in 1952. The instrument came into regular use in 1955, and has been employed in studying the mass spectra of a wide range of organic compounds, including hydrocarbons (2, 3), long chain alcohols (4, 5, 6), fatty acids (7, 8), rosin acids (9, 10), sterols and bile acids (11), and amino acids and peptides (12). Up to the present time about 450 mass spectra have been run of compounds with molecular weights of up to 619 (*n*-tetratetracontane). Since it was first put into use the instrument has been modified in several respects, leading to a considerable improvement in performance. Although there is still room for much improvement it appears that the present form of the instrument is satisfactory for the purpose it was designed. Very little information about instruments for the analysis of organic compounds of high molecular weight is to be found in the literature.

General description of the instrument

Fig. 1 shows the block diagram of the mass spectrometer. The analyzer tube is of the 180° semicircular type with a radius of 17.5 cm. A heated sample introduction system is employed in order to obtain complete vaporization of the compounds to be measured. For the scanning of mass spectra the magnetic current is varied, whereas the ion accelerating voltage is kept constant. This prevents influence of the voltage upon the ion yield. A photographic recorder with five galvanometers of different sensitivity is used. Because of the comparatively rapid response of the galvanometers, a wide range of masses can be recorded in a few minutes.

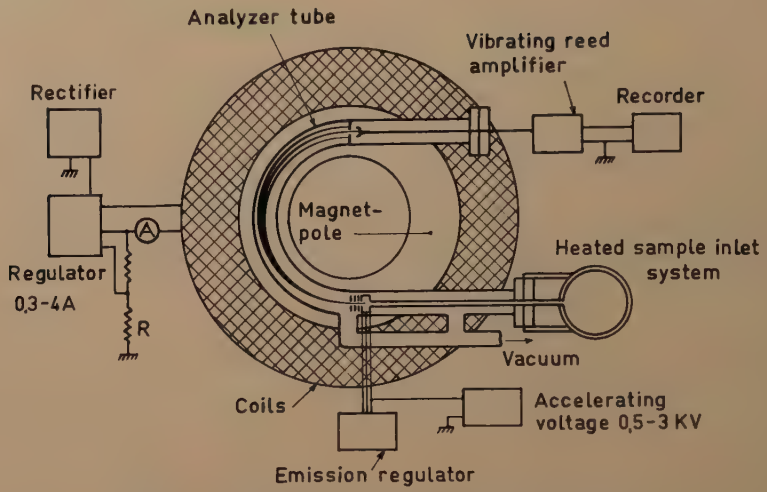

Fig. 1. Block diagram of the mass spectrometer.

Mechanical design

The magnet is placed upon a support of concrete with the pole gap in horizontal position at a height of 120 cm above the floor. A general view of the mass spectrometer is given in Fig. 2. The magnet pole pieces are ring-shaped and have an outer diameter of 45 cm. The pole gap is 4.5 cm and the cross-section of the gap is 4.5×10 cm. The ring-shaped pole surfaces have been handscraped to be flat and parallel within ± 0.01 mm. On each pole piece there are two coils which are connected in series. Each coil consists of 1960 turns of 1×1.75 mm wire. They are immersed in oil which is cooled by means of water circulating through coils of copper tubing. Fig. 3 shows a cross section of the magnet pole pieces and the coils. The total weight of the magnet is approximately 1600 kg.

The ion source, collector and the analyzer are made wholly from stainless steel with the exception of the focusing and slit plates which are made of nichrome. The ion source and the collector are mounted on flanges which are easily demountable from the analyzer. This also makes it possible to replace the filament easily. All parts of stainless steel are electropolished. The permeability of the stainless steel parts is below 1.005. The spectrometer tube (Fig. 4) has the cross-section shown in Fig. 5 and is bent to a mean radius of 17.5 cm.

The ion source is based on Nier's design (13). Fig. 6 shows the principal dimensions of the ion source, and a photograph of an ion source removed from the instrument is reproduced in Fig. 7. The exit slit has normally a width of 0.1 mm but the slit width can be varied over the range 0–1 mm. The resolving slit of the collector can be adjusted from the outside of the mass spectrometer tube by means of the device described by von Ubisch (14). It is possible to vary the width of the slit from zero to 1.5 mm and to tilt the slit up to $\pm 10°$ from the vertical position with the mass spectrometer in operation.

The tube is evacuated by a fore-pump, a booster pump, and a three-stage oil diffusion pump. A cold trap with dry ice and alcohol is used. The vacuum in the

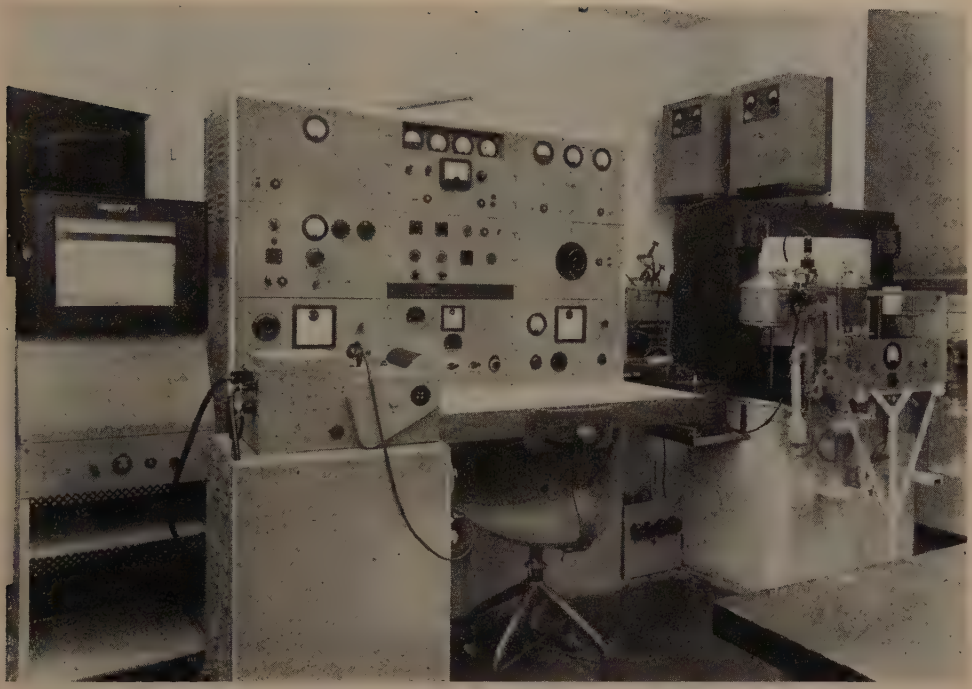

Fig. 2. General view of the mass spectrometer when put in operation in 1954.

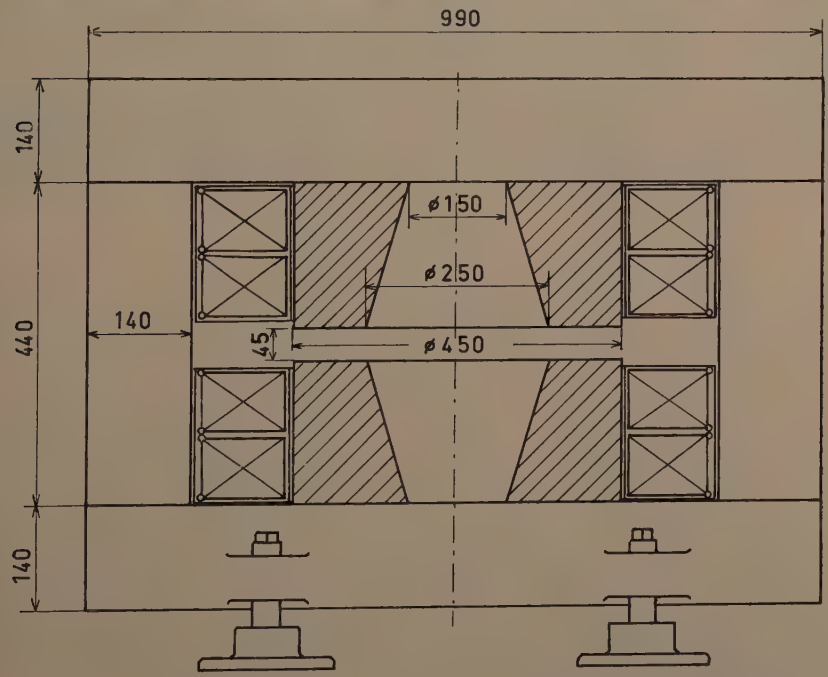

Fig. 3. The magnet with pole pieces and magnet coils shown in cross-section.

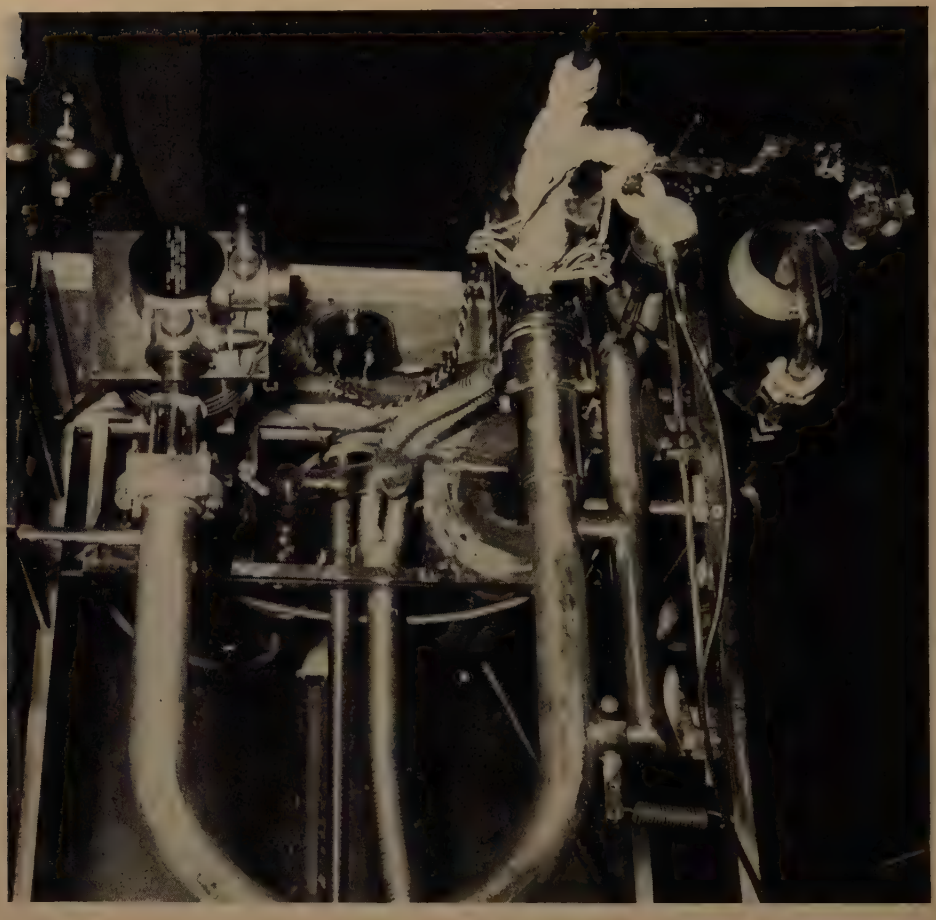

Fig. 4. Mass spectrometer tube withdrawn from the magnet.

analyzer is about 5×10^{-7} mm Hg, measured by a Penning type vacuum gauge (CEC type PHG-10). This cold-cathode gauge automatically cuts off the high voltage, the filament current and the oil diffusion pumps if the pressure becomes higher than 10^{-5} mm Hg.

The inlet system, introduction line, and gas leak are made of Pyrex glass, and can be operated at temperatures up to 400°C. The gas-inlet system is evacuated by a fore-pump and a mercury diffusion pump. Fig. 8 shows the inlet system which consists of a vapour reservoir with a molten gallium tube cut-off isolating the pumping system from the inlet system, and a ball joint cut-off isolating the vapour reservoir from the atmospheric air pressure when samples are introduced. The gas leak is of the molecular flow type and is placed near the reservoir.

The vacuum systems and the analyzer tube are mounted on a frame which can be removed from the magnet without breaking the vacuum in order to bake the tube for degassing purposes.

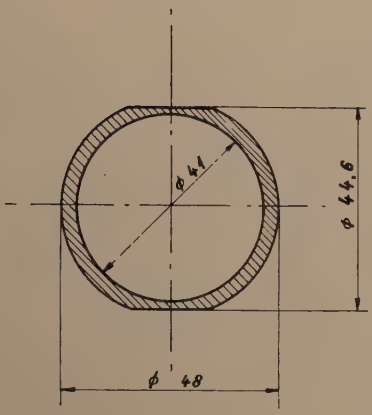

Fig. 5.

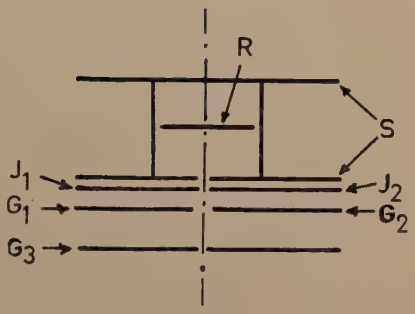

Fig. 6.

Fig. 5. Cross-section of mass spectrometer tube.

Fig. 6. Principal dimensions of the ion source. Separation of source elements: $S - J_1J_2 = 0.8$, $J_1J_2 - G_1G_2 = 3$, $G_1G_2 - G_3 = 6$ mm. Slit widths: $S = 0.5$, $J_1J_2 = 0.2$, $G_1G_2 = 0.8$, $G_3 = 0.1$ mm. The exit slit G_3 is grounded.

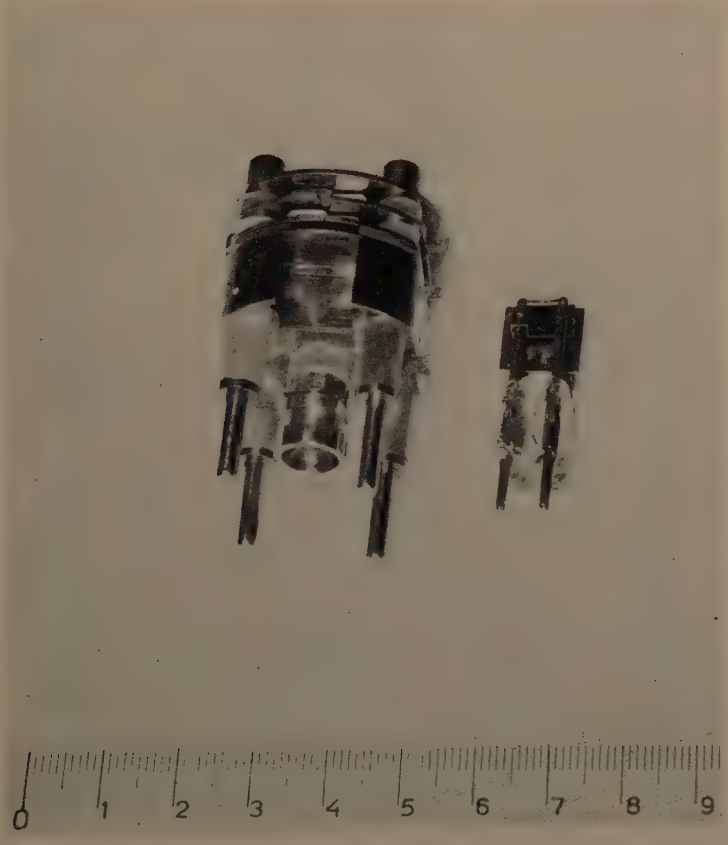

Fig. 7. Ion source removed from the instrument. Filament and screen shown to the right.

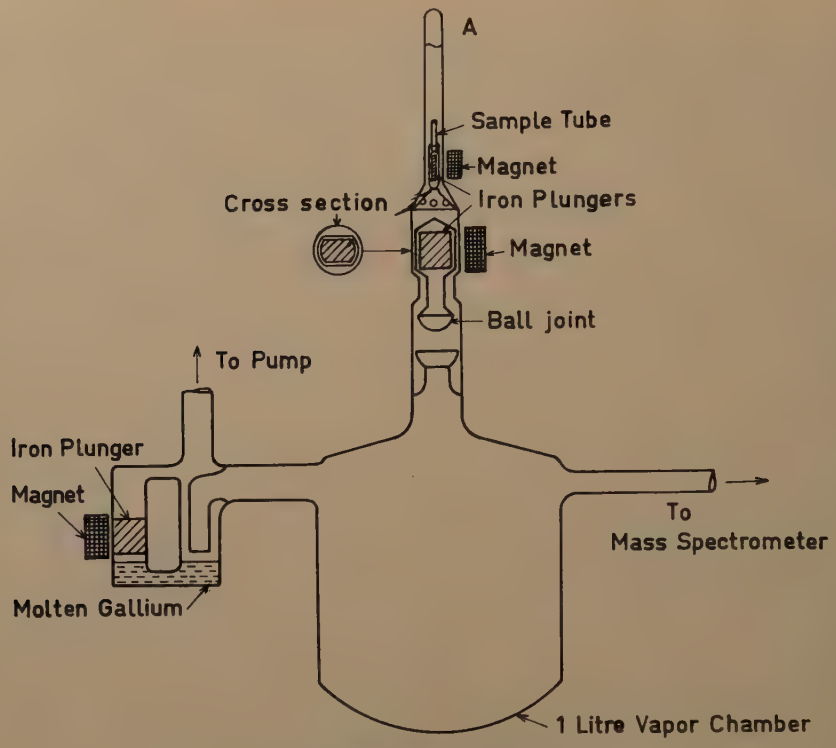

Fig. 8. Heated sample inlet system.

Electrical design

By adjusting the magnetic current over the range 0.3–4 ampere, the magnetic field is changed from 650 to 7700 gauss. This range is sufficient to cover a mass number range from about 4 to 580 at an ion accelerating voltage of 1500 volts as shown in Fig. 9.

A pre-stabilization of the three phase A.C. line voltage is obtained by means of two Philips type 7776/00 stabilizers coupled to two Variacs in *V*-connection. The rectifier circuit operates from this three-phase power source and six 872 hot-cathode mercury vapour rectifier tubes are used in the circuit. The regulator for the magnetizing current provides current stabilization. The magnetic current develops a voltage over a resistance R in Fig. 1, the voltage being compared with a reference voltage of 12 volts from a lead storage battery. The resistance R is made of manganine wire and is immersed in oil in a water cooled box. Eight 6AS7's are used as regulator tubes. The current of the regulator can be changed continuously within the range 0.3–4 ampere by a fifteen-turn Helipot potentiometer, which changes the reference voltage of the first stage in the amplifier. The regulated A.C. voltage from the Variacs is controlled manually to keep the voltage over the regulator tube below their maximum value. This is a somewhat inconvenient feature and it is intended to improve the instrument in this respect. Be-

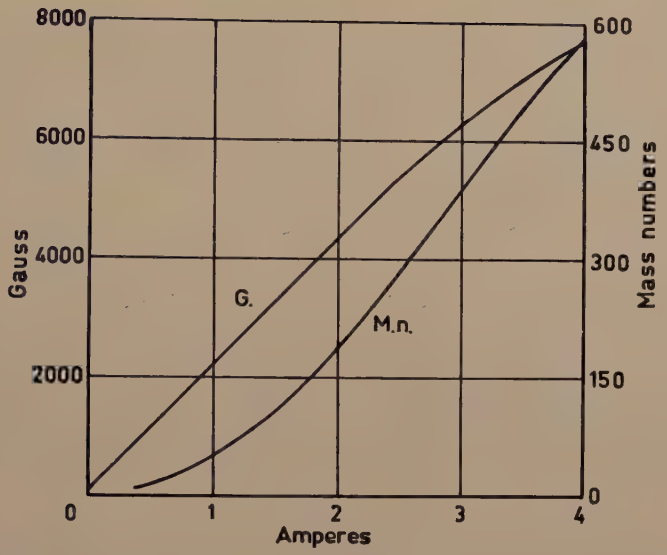

Fig. 9. Magnetization (G) and mass number (M.n.) as functions of magnetic current. Ion accelerating voltage 1500 volts.

cause excellent commercial magnet current supplies are now available, a detailed description of the present arrangement is not given. In any case it must be possible to keep the current constant to within less than ± 0.002 per cent when the line voltage changes ± 10 per cent.

The emission regulator used is based on same principle as that described by McKinney and his co-workers (15), except that the filament of the ion source is fed with a rectified current. Feeding the filament with low frequency alternating current may give an undesired modulation of the ion beam (16), and in the author's experience it also shortens the life of the filament which will be subject to strong vibration when high magnetic field strengths are used. The temperature of the ion source is not controlled. This is the most important weakness of the present instrument, and it is intended to add ion source temperature control in the near future.

The ion accelerating voltage is taken from a stabilized high voltage supply. In the output of the regulator a voltage divider is used so that the ion accelerating voltage can be varied between 500 and 3000 volts. However, the output voltage of the regulator can also be changed continuously by means of a small synchronous motor and a ten turns Helipot potentiometer. The voltage is stabilized to within ± 0.003 per cent for line voltage changes of ± 10 per cent.

The resolved ion masses are collected and the ion current is amplified by a vibrating reed amplifier (Model 36, Applied Physics Corporation, Pasadena, Cal., U.S.A.). The output of the amplifier is fed to a photographic recorder (AB Elektrisk Malmletning, Stockholm) which has five galvanometers with sensitivities in the ratios 1 : 3 : 10 : 30 : 60. The galvanometers are identical and their apparent sensitivities are determined by resistive networks. Each peak is recorded simultaneously by all five galvanometers, and the peak is read from the tracing of the most sen-

sitive galvanometer remaining on the paper. The D.C. sensitivity of the galvanometer with the amplifier employed is 3×10^{-2} volts per cm. This corresponds to 3×10^{-14} amperes per cm with an input resistor of 10^{12} ohm.

The recording paper measures 400×15 cm. The minimum time for scanning the mass range 12 to 300 is about four minutes. The accuracy of the peak height measurements is about 0.5 per cent when the deflection of the most sensitive galvanometer is greater than 30 mm.

Operation

As with all mass spectrometers, it is desirable to keep the ion accelerating voltage as high as possible in order to obtain high resolution and high ion intensity. However, a limit is set by the magnet current needed to reach the required mass range, which increases with the ion accelerating voltage according to the equation $M = k \cdot \frac{H^2}{E}$. For $H = 7500$ gauss the accelerating voltage should not exceed $E = \frac{860}{M} \times 10^3$ volts. Continuous scanning of a large mass range is possible by varying the magnetic current. In the case only a few neighbouring peaks in the spectrum is of interest, the accelerating potential can be varied with advantage.

A suitable quantity of the sample to be analysed (0.1–0.5 mg) depending on the molecular weight) is weighed up in a small glass tube (3 mm outer diameter) sealed at one end. The ball joint of the sample inlet system (Fig. 8) is put in the cut-off position, and the upper end of the introduction line cut open at A. After the sample tube has been introduced the inlet is sealed off at A. After pumping for about 5 minutes with filament current to the ion source cut-off, the ball joint is opened by energizing the magnet. The filament current is now switched on, and after pumping for another 10 minutes the gallium valve is closed. The heater for evaporating the sample is put in position over the sample introduction tube, and the sample heated to the temperature required to evaporate it. The glass reservoir and spectrometer inlet line are kept at a constant temperature in the range 100–250°, depending on the type of compound analysed. The temperature used depends on the type of compound analyzed during the whole sample introduction procedure.

A typical operation procedure for a long chain ester such as methyl *n*-hexacosanoate, ($CH_3(CH_2)_{24}COOCH_3$) is as follows. The ion accelerating voltage is held constant at 1800 volts. The gas is ionized by electrons with an energy of 70 eV. The m/e 12 peak is scanned one minute after the sample is completely vaporized. Five minutes are required to vary the magnet current from 0.55 to 3.8 amperes in scanning from m/e 12 to 410. The pressure in the inlet system, which is kept at a temperature of 200°, is calculated from PVT and is held at 0.05–0.1 mm Hg. When the sample has been measured a pump-out time of fifteen minutes reduces the peak at m/e 74 (the strongest peak) to less than 0.1% of the initial value for this compound.

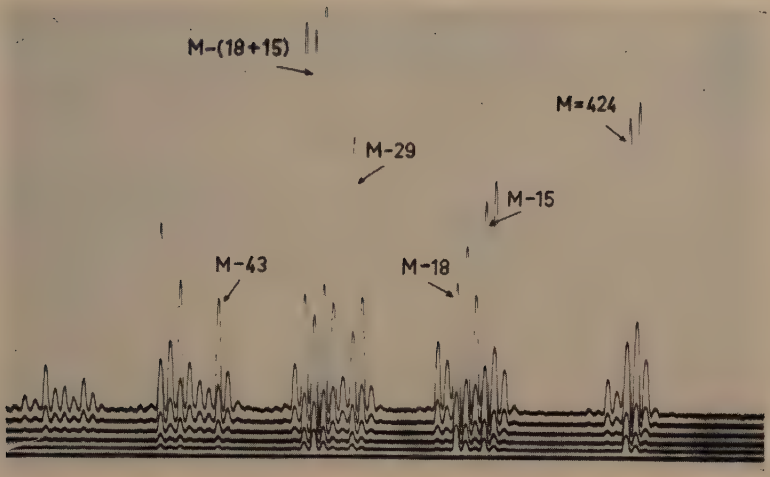

Fig. 10. High mass end of mass spectrum of urbanin.

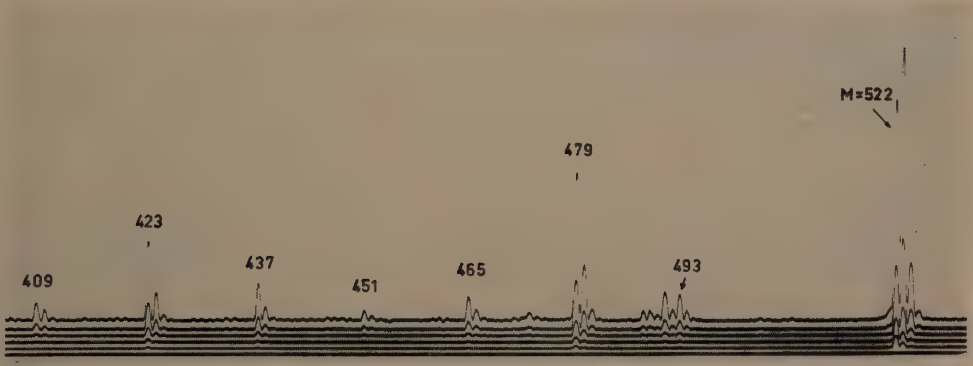

Fig. 11. High mass end of mass spectrum of methyl *n*-tetratriacontanoate.

Performance of the instrument

Unit resolution in a mass spectrometer is usually defined as existing when the valley between two peaks one mass unit apart has a height of two per cent of either peak if these are of equal height. In Figs. 10 and 11 are reproduced the high mass ends of the mass spectra of urbanin ($C_{30}H_{48}O$, $M = 424$) and methyl *n*-tetratricontanoate ($C_{35}H_{70}O_2$, $M = 522$) respectively. In the former case the valley between m/e 424 and 423 lies at a height less than one per cent of the m/e 424 peak. In the latter case it is seen that unit resolution is not reached under the conditions of the experiment. The slit widths in this particular experiment were, exit slit 0.1 mm and resolving slit 0.2 mm. The resolution may be improved by using narrower slits but this leads to lower ion currents, and the focusing of the ion beam becomes more difficult.

In organic structure analysis, however, it is often sufficient to be able to detect the rearrangement of one hydrogen atom, and this should be possible with the present instrument up to mass numbers exceeding 600.

SUMMARY

A 180° mass spectrometer with heated inlet system intended for organic analysis is briefly described. A complete spectrum can be obtained using about 0.5 μ mole of the compound to be analysed, and unit resolution extends up to m/e 500. The mass range 12–300 is scanned in four minutes.

ACKNOWLEDGMENTS

The construction of this instrument was made possible by grants from *Statens naturvetenskapliga forskningsråd, Statens Tekniska Forskningsråd, Atomkommittén* and *Statens Medicinska Forskningsråd* to Prof. E. Hammarsten, and from "*Stiftelsen Therese och Johan Anderssons Minne*" and *Statens Medicinska Forskningsråd* to Prof. E. Stenhagen. The author wishes to thank Messrs. E. Broberg and S. Warvenius for their cooperation in the construction of the mechanical parts of the instrument. The sample of urbanin was obtained from Prof. E. J. Corey, University of Illinois.

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An improved chromatographic method for halogenated acids and a discussion of their R_F- and R_M-values

By CHRISTOFFER RAPPE and NILS HELLSTRÖM

With 2 figures in the text

In connection with work on halogenated laevulic acids (1) attempts were made to separate these by means of paper chromatography. As developing solvent for halogenated organic acids, Hashmi and Cullis (2) have suggested propanol and ammonia, and several other authors (see Chittum, Gustin, McGuire and Sweeney (3) where references are collected) have used lower alcohols and ammonia or ethylamine. However, it was found that the laevulic acids reacted with the ammonia in the developing solvent (1), and therefore it was necessary to use an acid developing solvent. An aqueous mixture of *cyclo*-hexane, benzene and acetic acid appeared to be suitable for this purpose. Besides the different laevulic acids, some halogenated fatty acids were determined by this method.

Experimental part

Developing solvent, apparatus and procedure

The developing solvent was prepared in the following way: 60 ml of benzene and 30 ml of *cyclo*-hexane were shaken with water until the organic layer was saturated with water. The excess of water was separated, and the remaining liquid was filtered through filter paper moistened with the organic solvent, and thereafter 10 ml of acetic acid was added. Whatman's No. 1 filter paper was used, and about 2×0.004 ml of a 1 % ether solution (ca 50 μg) of each sample was applied. The bottom of the chamber was covered with water, but the chromatograms could be run without any equilibration period. The chromatograms were run for about two hours, and during this time the solvent front travelled 20–22 cm. After drying in air for a few hours the chromatograms were sprayed with a 0.04 % solution of bromophenol blue or bromocresol green in ethanol, cf. Linskens (4). The developed spots were generally round, but trichloroacetic acid, 2:3-dibromopropionic acid and 3:5-dibromolaevulic acid gave elongated spots.

Materials

The samples were of the following origin:

Commercial samples: Eastman: $CH_2BrCOOH$ (purum), CF_3COOH, $CH_3CHClCOOH$, CH_2ClCH_2COOH, $(CH_3)_2CHCHBrCOOH$; Fluka: CH_2JCOOH (puriss), CH_3CHBr-

COOH (purum); Light: $CH_3CH_2CHBrCOOH$, $CH_3CH_2CH_2CH_2CHBrCOOH$; Baker: $CH_2ClCOOH$ (p.a.); Merck: CCl_3COOH (p.a.); Kahlbaum: CH_2JCH_2COOH (recrystallized from water, m.p. 81–82.5°C); Matheson: $CH_3CH_2CH_2CHBrCOOH$; Kebo: $CH_3COCH_2CH_2COOH$ (purum, distilled three times in vacuo).

Prepared samples: according to Rappe (1): $CH_3COCHBrCH_2COOH$, $CH_2BrCOCH_2$-CH_2COOH, $CH_2BrCOCHBrCH_2COOH$; according to Cope, Clark and Connor (5): $CHCl_2COOH$; according to Abderhalden and Guggenheim (6): $CH_3CHJCOOH$ (m.p. 40–43°C); according to D'Ianni (7): $CH_2BrCHBrCOOH$ (m.p. 35–38°C); according to Rodionov and Gubareva (8): $CH_2ClCOCH_2CH_2COOH$ (m.p. 68.5–70.5°C); CH_2Br-CH_2COOH was prepared from acrylonitrile and hydrobromic acid (m.p. 59–61°C), compare Kendall and McKenzie (9): $CH_3COCHClCH_2COOH$ was studied as a mixture with $CH_3COCHBrCH_2COOH$ and $CH_3COCH_2CH_2COOH$, see Rappe (1).

Except $CH_3COCHClCH_2COOH$ all the samples were chromatographically pure.

Results and discussion

The R_F-values obtained according to the described method and the calculated R_M-values are collected in Table 1, where the R_F-values obtained by Hashmi *et al.* (2) are also given. The R_M-values are calculated according to Bate-Smith and Westall (10): $R_M = \log (1 - R_F)/R_F$.

The R_F-values are the mean values of at least three determinations, the individual determinations varied from the mean value by not more than ± 0.01 unit, except

Table 1. R_F- and R_M-values.

Acid	R_F-value according to Hashmi *et al.* (2)	R_F-value according to the described method	R_M-value
$CH_2ClCOOH$		0.23	+0.52
$CH_2BrCOOH$	0.45	0.30	+0.37
CH_2JCOOH	0.50	0.45	+0.09
$CHCl_2COOH$		0.15	+0.75
CCl_3COOH		0.05–0.12	+1.28–0.86
CF_3COOH		0	∞
$CH_3CHClCOOH$		0.59	−0.16
CH_2ClCH_2COOH		0.61	−0.19
$CH_3CHBrCOOH$	0.52	0.69	−0.35
CH_2BrCH_2COOH		0.67	−0.31
$CH_3CHJCOOH$	0.55	0.79	−0.58
CH_2JCH_2COOH	0.56	0.78	−0.55
$CH_2BrCHBrCOOH$		0.71	−0.39
$CH_3CH_2CHBrCOOH$	0.60	0.84	−0.72
$CH_3CH_2CH_2CHBrCOOH$	0.70	0.90	−0.95
$(CH_3)_2CHCHBrCOOH$	0.70	0.89	−0.91
$CH_3CH_2CH_2CH_2CHBrCOOH$		0.92	−1.06
$CH_3COCH_2CH_2COOH$		0.22	+0.55
$CH_3COCHClCH_2COOH$		0.44	+0.10
$CH_3COCHBrCH_2COOH$		0.54	−0.07
$CH_2ClCOCH_2CH_2COOH$		0.30	+0.37
$CH_2BrCOCH_2CH_2COOH$		0.38	+0.21
$CH_2BrCOCHBrCH_2COOH$		0.62	−0.21

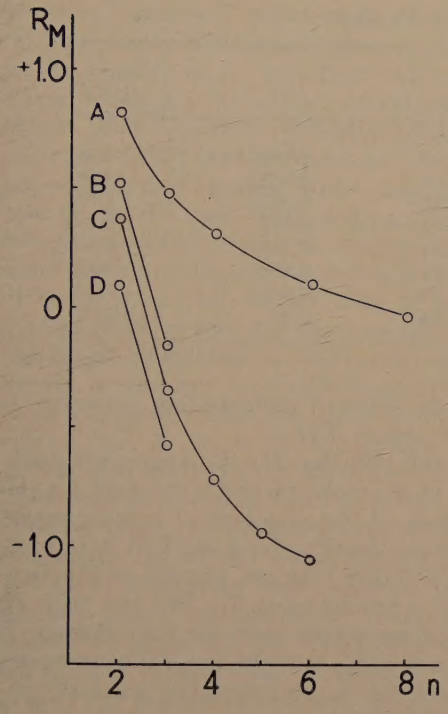

Fig. 1. R_M-values plotted versus number of carbon atoms (A) monocarboxylic acids, (B) chloro-, (C) bromo- and (D) iodosubstituted acids.

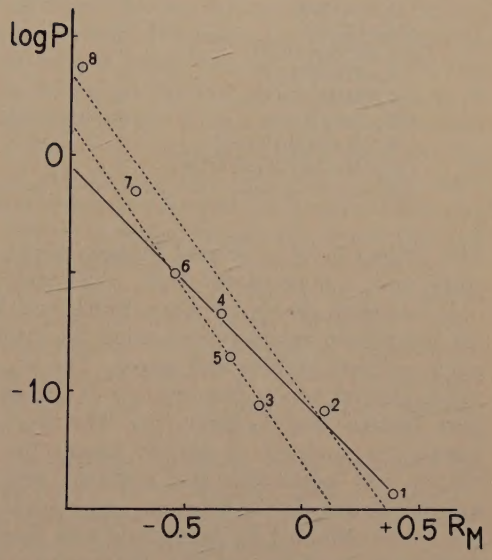

Fig. 2. log P plotted versus R_M. The full line has the theoretical slope -1.

for trichloroacetic acid. It was found that the R_F-value of this acid depended on added amount: increasing amount of acid gave increasing R_F-value, compare Renard (11).

A comparison with the values obtained by Hashmi *et al.* (2) showed that their values are confined within narrower limits, 0.45–0.70, than those found here, 0.29–0.90. Nevertheless different isomers had about the same R_F-values here as found by Hashmi *et al.* (2). The only isomers which could be separated were the different halogenolaevulic acids. It is interesting to notice that the difference of the R_M-values between 5- and 3-isomers is the same for the chloro- and bromolaevulic acids (chloroisomers = 0.27, bromoisomers = 0.28). It is worth mentioning that the influence of a second halogen atom, as in 3:5-dibromolaevulic acid, exerts an appreciable influence upon the R_F-value compared with those of either of the monosubstituted acids.

Like Hashmi *et al.* (2) the present authors found that the R_F-values were a function of the molecular weight, i.e. increasing length of the carbon chain and increasing weight of the halogen atom gave higher R_F-values, compare Macek and Michalec (12). These authors also state, that the influence of the first halogen atom introduced is rather small, but with reference to the laevulic acid and the 5- and especially the 3-halogenated laevulic acids this is not valid. The influence of a second and a third halogen atom at the same carbon atom is also evident, cf. Macek and Michalec (12).

Table 2. R_M- and log P-values.

Acid	No. in Fig. 2	R_M	P	log P
$CH_2BrCOOH$	1	+0.37	0.0362	−1.44
CH_2JCOOH	2	+0.09	0.0831	−1.08
CH_2ClCH_2COOH	3	−0.19	0.0873	−1.06
$CH_3CHBrCOOH$	4	−0.35	0.212	−0.67
CH_2BrCH_2COOH	5	−0.31	0.140	−0.85
CH_2JCH_2COOH	6	−0.55	0.319	−0.50
$CH_3CH_2CHBrCOOH$	7	−0.72	0.708	−0.15
$CH_3CH_2CH_2CHBrCOOH$	8	−0.95	2.32	+0.37

The influence of a second halogen atom at an adjacent carbon atom, however, is small with the propionic acids (0.69 and 0.67 versus 0.71).

It has been shown by Bate-Smith and Westall (10) that the R_M-values are made up of additive values representing the groups in the molecule and a constant for the given solvent system and paper. The R_M-values of the members of a homologous series plotted versus the number of carbon atoms usually give a straight line, compare Lederer and Lederer (13). The R_M-values found here are plotted in Figure 1 versus the number of carbon atoms for the α-bromo acids (C). The two pairs of chloro- and iodo-acetic and α-chloro- and α-iodopropionic acids are also plotted (B and D). Evidently the α-bromo compounds do not give a straight line. For comparison the curve obtained by Isherwood and Hanes (14) for the corresponding non-halogenated acids is given too (A). Isherwood *et al.* (14) also found that these acids do not form a straight line. According to Lederer *et al.* (13) this could be due to adsorption. Isherwood *et al.* (14) used propanol and ammonia as developing solvent, hence their R_F-values are related to ammonium salts.

Owing to the theory that the R_M-values are additive throughout a series, the Δ_{CH_2} R_M-values between a monohalogenated acetic acid and the corresponding α-halogenopropionic acid should be the same in the cases of chloro-, bromo- and iodocompounds. Evidently this is the case, cf. Figure 1.

The Δ R_M-values for the chloro and the corresponding bromo acids can here be studied in five pairs. The values are $\Delta_{Cl,Br} = 0.15$ (acetic acids), 0.19 (α-propionic acids), 0.12 (β-propionic acids), 0.17 (3-laevulic acids), 0.16 (5-laevulic acids). Three pairs of bromo- and iodocompounds are studied: $\Delta_{Br,J} = 0.28$ (acetic acids), 0.23 (α-propionic acids), 0.24 (β-propionic acids). The first series gives the mean value 0.16 and the second 0.25. The difference seems to be significant. The fact that $\Delta_{Cl,Br} < \Delta_{Br,J}$ might be due to the differences in for instance atomic volume, dipole moment of the functional group and/or polarisability of the halogen atoms.

Martin (15) has given the formula for the R_F-value and the partition coefficient (α) between water and an organic solvent:

$$\frac{1-R_F}{R_F} = \frac{\alpha}{k},$$

where k is a constant depending on paper and solutes. In the case here no partition coefficients have been determined. Smith and White (16), however, determined the partition coefficient P of some halogenated organic acids in the systems water and

benzene, water and toluene and water and chloroform. They considered the degree of dissociation into ions in the aqueous layer and association into double molecules in the organic layer, and they determined the coefficient P (= (concentration of single molecules in organic layer)/(concentration of single molecules in aqueous layer)). This P-value is evidently independent of the total amount in the two phases. They also found that the quotient (q) of P in regard to two solvent pairs, e.g. chloroform–water and benzene–water, was the same for the different acids. Supposing this also to be the case when comparing their results (with benzene–water) with the two phases which might be represented in this investigation, it appeared to be of some interest to see if a simple relation exists between the R_M-values and the P-values of Smith *et al.* (16). As a matter of fact a linear correlation between $\log P$ and R_M is in accordance with the experimental facts, see Table 2 and Figure 2. At first sight one might be inclined to express the relation by two nearly parallel lines (dotted), one for the α- and one for the β-compounds, but a statistical treatment shows this not to be significant. Further, there is some doubt about the R_M-value for α-bromo-*n*-valeric acid, No. 8; its R_F-value is 0.90, therefore the error of R_M might be appreciable, cf. Lederer *et al.* (13). A statistical treatment gives for all acids the slope -1.32; when α-bromo-*n*-valeric acid is excluded, the slope is -1.12. Neither of these values however proved to be significantly different from the value -1. Due to the assumption made, we set the actual partition coefficient for the chromatographic system, $F = 1/qP$. The theory then predicts: $\log q \cdot P = -1 \cdot R_M + c_1$ or $\log P = -1 \cdot R_M + c_2$ where c_1 and c_2 are constants. Therefore, considering the assumptions made and the statistical treatment, the experimental results are in accordance with the theory. On account of difficulties in defining the phases actually present in paper chromatography the procedure used here might be of wider interest and value.

SUMMARY

A new developing solvent for paper chromatography of halogenated aliphatic acids has been prepared. It has been used for determination of the R_F-values of 23 acids. From the R_F-values the R_M-values are calculated. These are compared in regard to homology, isomery, kind and number of halogen atoms present. They are also compared with partition coefficients of a similar system. Within the limits of experimental error the theoretical relation between the partition coefficient and the R_M-value is confirmed.

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